
Microbial Ecology of Aquaculture Sediments

By

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Declaration

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution, and to the best of my knowledge contains no copy or paraphrase of material previously published or written by any other person except where due reference is made in the text of the thesis

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22/6/2005

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Abstract

Sediment bacterial communities were studied at two Tasmanian salmon farm sites and adjacent unimpacted reference sites. Microbial biomass and total diversity were investigated as well the diversity and population density of the *Cytophaga-Flavobacteria-Bacteroides* group (CFB) and beta-proteobacterial ammonia oxidizers (AOB). The study aimed to develop a conceptual understanding of microbial community dynamics in response to disturbance and to assess the efficacy of farm fallowing practices in allowing sediment bacterial communities to recover from organic loading.

Sediments from two salmon farms were studied over two full farm production cycles of 12 months each. Each cycle consisted of a nine-month stocking period, during which organic loading occurred, followed by a three month fallow period, during which no loading occurred. To represent the range of sediment conditions prevalent at Tasmanian salmon farms, farms with differing sediment conditions were chosen. Sediment at farm 1 was coarse grained, while at that at farm 2, sediment was fine grained. Sampling was conducted at the beginning of each cycle and at the end of each period within a cycle.

Bacterial numbers increased as farming and organic loading progressed through the farm cycle and declined during the fallowing period, although not to pre-stocking levels. Bacterial numbers ranged between approximately 2×10^8 and 3×10^9 cells/g sediment and were generally higher at cage sites than reference sites.

Six, 16S rRNA gene clone libraries were constructed, comprising more than 600 clones sequences, from both cage and reference sites. These revealed that both cage and reference site sediments at both farms showed a very high level of diversity. Reference sites were dominated by delta and gamma-proteobacteria and CFB group bacteria. Cage site sediments also showed large numbers of these phylotypes, as well as members of the alpha and epsilon-proteobacteria. Diversity and coverage indices indicated that the diversity of all sediments studied was much greater than that detected in this study, despite a large sampling effort. All clone libraries were shown to be statistically different from one another, further supporting the idea that coverage

was low. Many phylotypes did not group with cultured bacteria, but grouped with other environmental clones from a wide array of marine benthic environments. Clone libraries indicated the presence of a large number of bacterial types, including the Myxobacteria. Although thought to be unable to grow under marine conditions, the large number of Myxobacteria clones found in this study further supports the idea that this may not be the case.

Denaturing gradient gel electrophoresis (DGGE) showed that bacterial communities shifted both in response to farm loading and the cessation of this loading.

Communities also shifted frequently at reference site communities, indicating the highly dynamic nature of sediment bacterial communities and the possibility of a seasonal effect. Although bacterial communities did shift again during the fallowing period this shift was not necessarily evidence of a return to pre-loading communities. The complexity of community shifts and their interpretation could be attributed to the vast functional redundancy of bacterial groups. Respiration studies indicated that cage site sediments were as resilient and as diverse as reference site sediments.

CFB and AOB communities too showed shifts and counter shifts with organic loading and fallowing. Again the exact nature of the shifts was difficult to elucidate: communities did not show a simple shift/counter-shift response to farm loading and fallowing. Real-time PCR showed that CFB numbers increased with farming and decreased with fallowing. The response of the CFB group to organic loading was typical of that expected of an opportunistic group. Real-time PCR analysis of the AOB showed that farm loading had little effect on their numbers. The AOB were absent from clone libraries, but analysis of DGGE band sequences showed that a diverse AOB community was present at both farms at all times during the study, thus maintaining at least the potential for the coupled nitrification/denitrification process.

Bacterial community shifts and response to perturbation are difficult to interpret, as a result of the massive functional redundancy exhibited by bacteria. The sediment bacterial community though, appeared to be determined by sediment environmental parameters. This is in contrast to sediment infaunal communities, which tend to respond according to the equilibrium theory. Bacterial biomass and diversity

responded to farm fallowing practices, thus these practices appeared efficacious in maintaining diverse, resilient sediment bacterial communities.

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Chapter 1

Introduction

Chapter 1 Introduction

Organic enrichment is the most widely encountered type of marine pollution and is increasing as population settlement and marine industries grow. As awareness of anthropogenic organic inputs has grown, coastal zones (and in particular estuarine zones) have become increasingly important in discussions regarding impact and recovery. An understanding of ecosystem function and response to anthropogenic input is required to ensure the sustainability of coastal values. Microbial communities play a pivotal role in organic carbon mineralisation in coastal ecosystems.

Decomposition is driven by microbial activities and is fundamental to ecosystem function (Zak, Willig, Moorhead, Wildman, 1994). Degradation of environmental contaminants is also performed by microbial communities. An understanding of the microbial response to anthropogenic input is necessary to ensure sustainability of coastal zones.

1.1 Sediment microbiology

Sediments are unique environments for several reasons: they are often permanently overlain or perfused with water and the inhabitants are necessarily linked to their chemical environment, to the extent that the sediment chemical environment is dependant on benthic metabolism, and abundance and community composition is driven by the chemical environment (Giblin, Foreman, Banta, 1995). The result of this association is that sediment communities possess well developed feed-back loops between the living and chemical components of the community and are able to respond quickly in dynamic situations (Duplisea, 1998; Giblin, Foreman, Banta, 1995).

Prokaryotic microbial biomass and diversity in soils and sediments far exceeds that of eukaryotic organisms; one g of sediment may contain more than a billion micro-organisms of more than 10^4 different species (Rossello-Mora, Amann, 2001; Torsvik, Sorheim, Goksoyr, 1996). The sediment microbial community is of fundamental importance to system function, determining nutrient cycling, decomposition and energy flow (Wardle, Giller, 1996). Bacteria are primarily responsible for the cycling of nutrients and therefore contribute to a system's assimilative capacity.

Oxygen levels in sediments are limited by its solubility in water and are influenced by: the dissolved oxygen levels of the overlying water; mass transfer across the diffusive boundary layer, diffusion in pore water; the concentration of organic carbon in sediments (which affects respiratory rates); the concentration of reduced inorganic compounds. The presence of organic matter that is able to be aerobically respired results in sediments becoming more anoxic with depth (Nealson, 1997). The depletion of oxygen results in the utilization of alternative electron acceptors (NO_3^- , NO_2^- , and Mn and Fe oxyhydroxides, SO_4^{2-}). These oxidants are consumed in order of their decreasing redox potentials. In undisturbed sediments this process results in the formation of a series of stable gradients (Nealson, 1997) that are determined by the organic input, microbial metabolic capacities and the system's geochemical composition. In many sediments though, and certainly in the shallow in-shore sediments that are the subject of this study, a simple stratification based on redox/depth is not possible. Bioturbation (Heilskov, Holmer, 2001; Wu, Tsutsumi, Kita-tsukamoto, Kogure, Ohwada, Wada, 2003) and sediment mixing due to climatic events ensure that these sediments are not undisturbed in the sense alluded to above. They are, instead, very dynamic systems.

Microbial activity in marine sediments is determined by geographic position, sedimentation rate, sedimenting material, temperature and sediment chemical conditions. Sediment communities respond rapidly to altered nutrient input (Battersby, Brown, 1982; Cloern, 2001; Paerl, 1998; Paerl, Dyble, Maoisander, Noble, Piehler, Pinckney, Steppe, Twomey, Valdes, 2003). Eutrophication may lead to increased microbial production (Battersby, Brown, 1982; Rysgaard, Risgaardpetersen, Sloth, Jensen, Nielsen, 1994), oxygen depletion and production of toxic metabolites. Hypoxia and anoxia may lead to the replacement of aerobic species with microaerophilic (e.g. *Beggiatoa* spp.) and anaerobic species and as a result, alterations to ecosystem composition and function (Paerl, 1998). Microbes may respond opportunistically to nutrient input, as is the case with many algal species when nutrient enrichment extends to the water column (Richardson, 1997) or with heterotrophic organisms colonising particulate aggregates (Cottrell, Kirchman, 2000). The sediment environment may also become more homogeneous as it becomes more extreme, thus limiting the diversity it can support (Battersby, Brown, 1982).

1.2.2 Development of microbial communities

Two schools of thought exist as to the determination of community structure in macro ecology. The most dominant holds that within a given habitat, populations compete to fill ecological niches. With time, the most efficient populations become established and less efficient populations become displaced. Community structure, then, develops over time to reach stability if environmental conditions (and niches) remain relatively constant. This is known as equilibrium theory (Reice, 1994). Ecosystems vary in the number of niches they have to offer and consequently the range of inhabitants they can support. Some extreme systems may also possess more niches than are filled, as the number of populations with the physiological capabilities of withstanding the extreme environment may be limited. Alternatively to equilibrium theory is the idea that community structure is determined by its environment. That “the presence or absence of a given taxonomic group results from random colonization processes and variability in the environment” (Reice, 1994).

Equilibrium theory holds that the succession of communities proceeds through primary colonisation, succession and finally the formation of a climax community (Atlas, Bartha, 1998). Primary colonization is the colonization of a system by the first organisms, succession refers to the replacement of the primary organisms by new organisms as the system changes and the climax community refers to the achievement of a stable community and the end of succession. It is possible for the climax community to comprise the primary colonisers, but more common is the occurrence of multiple/continuous succession events. In fact, in terms of microbial communities it is very rare for a single climax community to be achieved. Instead successional population changes occur regularly to maintain relatively stable systems over time. Rather than reaching a classic climax community these systems are continually in a state of flux, whereby disturbances maintain the successional process, which occurs continuously. Environments that continually experience low level disturbances may harbour the highest diversity (Wardle, Giller, 1996).

Successional processes are caused by modifications to the system by both the resident organisms and by external forces. The exact nature of the causative factors of shifts in communities is often difficult to elucidate and often involves a cascade of events.

For example, increased sedimentation may increase the nutrients supplied to a community, heterotrophic bacteria may thrive and create anaerobic zones, creating a new niche for anaerobic organisms. After the cessation of sedimentation the reverse process may occur. Rapid generation times and a high level of functional redundancy in micro-organisms further complicate the successional process. In continually changing systems; a temporal niche may not be filled by the same organism every time it arises, but the organism that fills it will have the same function.

The fact that microbial communities are not static and that a true climax community is not reached does not imply that they are not stable (resistant and resilient). The very nature of complex microbial communities (rapid generation times and functional redundancy) often results in a homeostatic reaction by the community when disturbed. Catastrophic events are possible (although very rare in microbial communities) though, and result in the creation of entirely new habitats and primary colonisation. During succession there is often a higher level of diversity than when more stable communities are reached (Pearson, Rosenberg, 1978), but the relationship between diversity and stability is a contentious one.

Two opposing thoughts have evolved regarding the impact of diversity on ecosystem function and stability. One idea expounds that increased species diversity improves both function and stability (Naeem, Thompson, Lawler, Lawton, Woodfin, 1995; Tilman, 1996), while the other maintains that it is the functional role of the organisms present that determines ecosystem function and stability, rather than the diversity of individual species present (Hooper, Vitousek, 1997). These ideas regarding diversity and its effect on ecosystem function have developed using terrestrial, macro-eukaryotic models and have been poorly investigated in microbial communities.

1.2.3 Concepts of diversity in microbial communities

Historically the unit used to measure diversity has been the species, but the species concept with regard to prokaryotes is contentious (Rossello-Mora, 2003; Rossello-Mora, Amann, 2001). It may be more useful to think of diversity comprising three components: genetic, functional and taxonomic diversity (Forney, Zhou, Brown, 2004; Solbrig, 1991). Traditionally then, research has focused on taxonomic

diversity, but increasingly this focus has shifted to genetic and functional diversity, (for example (Amann, Ludwig, Schleifer, 1995; Bano, Hollibaugh, 2002; Bowman, McCuaig, 2003; Bowman, Rea, McCammon, McMeekin, 2000; Bowman, McCammon, Gibson, Robertson, Nichols, 2003; Purkhold, Wagner, Timmermann, Pommerening-Roser, Koops, 2003; Ravenschlag, Sahm, Amann, 2001; Ravenschlag, Sahm, Pernthaler, Amann, 1999)).

There is a close relationship between sediment microbial community members and ecosystem processes, making sediment communities ideal for investigating community-level responses to stress and disturbance. In soils, decomposition rates are not generally affected by diversity (Wardle, Giller, 1996), but the concept of keystone species needs to be considered. Keystone species are those species that perform a specific task integral to the stability of a system, and that is not fulfilled by a wide range of organisms, but which have a level of functional redundancy that may compromise the filling of their niche if environmental conditions are altered. Even this concept is contentious and continually evolving. Recently, the ammonia-oxidising bacteria may have been considered a keystone group, sensitive to environmental changes (especially those concomitant with organic loading and hypoxia) (Rysgaard, Risgaardpetersen, Sloth, Jensen, Nielsen, 1994), but the discovery of organisms capable of the ANAMMOX reaction (Kuypers, Sliekers, Lavik, Schmid, Jorgensen, Kuenen, Damste, Strous, Jetten, 2003) has cast doubt on this idea. It may be that microbes are so functionally redundant and regenerate so rapidly that all niches will always, eventually be filled.

1.2 Study context and research objectives

An understanding of ecosystem response to anthropogenic perturbation is necessary to ensure the sustainable development of near-shore coastal areas. Eutrophication is the most common form of pollution to near-shore marine areas. Alterations to microbial communities and their function may lead to wider impacts on microbial-loop/food-web interactions and ultimately impacts on invertebrates and fish as well as nutrient cycling and oxygen fluctuations (Paerl, Dyble, Maoisander, Noble, Piehler, Pinckney, Steppe, Twomey, Valdes, 2003). Even though microbial communities play a pivotal role in carbon cycling processes (Plante, Wilde, 2004), their response to disturbance

and the potential impacts of this response on ecosystem processes is poorly investigated and understood. In order to predict the system-level implications of microbial community response to disturbance it is first necessary to understand the community dynamics themselves. The impacts of disturbance, the rate of recovery and resultant successional changes will be dependant upon the factors that determine community structure and resilience.

Aquaculture is a rapidly growing industry (Love, Langenkamp, 2003) and a known point source of eutrophication in coastal marine systems (Findlay, Watling, Mayer, 1995; McGhie, Crawford, Mitchell, O'Brien, 2000; Wu, 1995). As such it provides an ideal model to study microbial responses to organic loading and its cessation. This work was undertaken as part of an Aquafin Cooperative Research Centre (CRC) study into the effects of salmon farming on the Tasmanian marine environment (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). Two salmon farms with differing sediment conditions (see chapter 1.4) were used to explore the response of the microbial community to organic input and its cessation during fallowing.

Studies of invertebrate fauna in these sediments have shown that community structure is determined by factors similar to those expounded by the equilibrium theory. That is, the community was shown to return toward its former (presumably equilibrium) state after cessation of the disturbance (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). It was hypothesized that the microbial community would behave in a similar manner. That is, that it would shift in response to organic loading and return toward its equilibrium state following removal of the loading. In order to test this hypothesis several analyses were performed on fish-farm sediments to answer the following questions:

- 1) What is the microbial biomass and how does this change after organic loading?
- 2) Which bacterial phylotypes are present in Tasmanian coastal sediments and how does this diversity shift after disturbance by increased organic input?
- 3) How does the bacterial community respond to the cessation of organic loading??

- 4) How does a keystone bacterial group respond to organic loading and its cessation?
- 5) How does an opportunistic bacterial group respond to organic loading and its cessation?

These specific questions were designed to explore the more general idea that microbial communities behave in a similar way to traditional ecological communities (macro) when subjected to disturbance. Organic disturbance causes sediment infaunal communities to initially increase in biomass (question 1) and to initially, briefly increase in diversity and then to decrease in diversity, (question 2 and 3). It also causes keystone species to be displaced (question 4) and opportunistic species to become dominant (question 5). All of these processes have been shown to be reversible in invertebrate faunal species of these sediments with the cessation of the disturbance (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004).

1.3 Experimental Approach

A variety of approaches and techniques have been used to study microbial communities generally, and in sediments. The following section discusses these approaches and outlines the reasons for the choice of techniques used in this study.

1.3.1 Molecular analysis of microbial communities

The study of microbial communities is focussed at achieving three goals:

1) To define population dynamics in communities, 2) define physicochemical characteristics of microenvironments and 3) understand the metabolic processes carried out by micro-organisms in specific habitats.

The chemical cycling of the biosphere is largely dependant upon microbial activity, yet because of a reliance on culture-based techniques, relatively little is known about the composition of microbial communities (Amann, Ludwig, Schleifer, 1995; Pace, 1995). Because many species have proven resistant to cultivation, cultivation based methods are said to only sample 0.1 – 1% of the microbial population (Amann, Ludwig, Schleifer, 1995). Traditionally micro-organisms have been described by

their phenotype (observable cellular properties including morphology, cellular structure and physiological parameters). An alternative approach is the phylogenetic investigation of microbes and microbial communities. Molecular techniques, which do not require the laboratory cultivation of organisms, have been developed and utilized to characterise the bacterial communities of many environments (Muyzer, Ramsing, 1995). Described below are some of the methods in which molecular markers, particularly 16S rRNA genes, are utilised to detect and characterise microbial communities.

16S rRNA genes as biomarkers

The use of biological markers, which include lipids and genetic markers, to investigate microbial communities has provided a new approach to studies in microbial ecology (Liesack, Janssen, Rainey, Ward-Rainey, Stackebrandt, 1997). Of the genetic markers employed for such studies, genes that provide information regarding the presence or absence of a phenotypic trait (gene expression) and those indicating phylogenetic relatedness of organisms have been utilised (Liesack, Janssen, Rainey, Ward-Rainey, Stackebrandt, 1997). The small subunit ribosomal genes (16S rRNA gene in prokaryotes) are the most extensively used, for several reasons: 1) they are present in all organisms, 2) they contain conserved and variable regions, 3) they contain enough sequence information to be used as phylogenetic markers, 4) rRNA is a dominant cellular constituent and 5) the genes are not transferred horizontally (Heuer, Smalla, 1997; Liesack, Janssen, Rainey, Ward-Rainey, Stackebrandt, 1997; Muyzer, Ramsing, 1995; Woese, 1987). These features ensure that it is possible to make inferences regarding phylogeny, based on sequence comparisons to known organisms. Although some phylotypes do occur in monophyletic clusters (e.g. β -proteobacterial ammonia-oxidising bacteria) it is usually very difficult to infer function from 16S rRNA sequences (Fuhrman, 2002; Ward, Bateson, Weller, Ruff-Roberts, 1992), although such an inference is often a goal of studies into microbial ecology.

1.3.1.1 rRNA techniques to monitor diversity in microbial ecology

Analysis of natural microbial communities may be motivated towards several ends, ranging from attempting to catalogue the community in its entirety to assessing a

snap-shot of the community at a given time. Discussed below are the techniques of clone library assemblage and several polymorphism based (fingerprinting) techniques.

Clone libraries

Perhaps the most sensitive approach to assessing microbial diversity is assembling 16S rDNA clone libraries of microbial assemblages. After nucleic acids have been extracted from environmental samples, clone libraries may be constructed in three ways (shot gun cloning, cloning of rRNA after reverse-transcriptase-PCR and direct cloning of PCR amplified 16S rDNA), the most popular of which is the cloning and sequencing of 16S rDNA amplification products. After PCR amplification of community 16S rDNA, fragments can be cloned into commercially available sequence-ready vectors (Theron, Cloete, 2000). Clones may then be screened (for example using RFLP) and sequenced or simply sequenced. Sequences can then be compared to one another or other sequence databases (e.g. Genbank) and community diversity assessed. In complex communities, it is necessary to sequence very large numbers of clones in order to gain an insight into community diversity (Kemp, Aller, 2004). Clone libraries, then, offer the highest resolution in assessing natural microbial community diversity, but are very time consuming and expensive.

Fingerprint Techniques

Genetic fingerprint techniques provide a means to analyse successional changes in natural microbial communities (Muyzer, 1999), a major aim of many microbial ecological studies. Clone libraries are too laborious to provide statistically meaningful data regarding microbial community change in most studies. Fingerprint techniques provide a rapid means to simultaneously analyse many samples in a statistically valid manner, enabling the assessment of community change along environmental gradients or over time. Genetic fingerprint techniques seek to provide a pattern representing microbial community diversity based upon polymorphisms in, and the physical separation of, unique community nucleic acids (Muyzer, 1999). Below, I will describe various methods by which fingerprinting techniques are utilised to study the diversity of natural microbial communities. All of the methods discussed are said to be indirect methods, because nucleic acids are extracted and amplified prior to analysis (Muyzer, 1999). Direct fingerprinting methods will not be discussed

because such methods are currently not suitable for long term monitoring of sediment communities.

Denaturing gradient gel electrophoresis (DGGE)

DGGE was first used to analyse natural microbial communities by Muyzer *et al.* (1993). In DGGE, DNA PCR fragments of the same length, but different sequences can be separated according to their melting properties (Heuer, Smalla, 1997). The melting temperature, T_m , of the fragment is determined by its sequence. The DNA is electrophoresed through a linearly increasing gradient of denaturants, these may be chemical or temperature based. The fragments remain double stranded until they reach the conditions that cause melting of the lower melting domains within the strand. The branching of the molecule caused by this melting sharply decreases the mobility of the molecule through the gel. A GC-rich clamp on one end of the primer set prevents the complete melting of the fragment. DGGE has become popular in the analysis of environmental samples, since it allows the generation of fingerprint snapshots of complex microbial communities, and the comparison of large numbers of samples (Muyzer, Ramsing, 1995). DGGE also has the potential to provide phylogenetic information if bands are excised and sequenced or gels are hybridized with specific probes. DGGE is, though, not without its caveats: similar melting behaviour of different sequences may produce bands of the same mobility, complex communities may produce gels too complex to resolve bands and band doublets may hinder gel interpretation (Jackson, Churchill, 1999; Jackson, Roden, Churchill, 2000; Kisand, Wikner, 2003) .

Single Stranded Conformational Polymorphism (SSCP)

In SSCP, single stranded DNA fragments are electrophoresed on a non-denaturing gel. The conformation shape taken by the single stranded DNA influences its electrophoretic mobility, allowing separation of fragments. Initially, DNA fragments are denatured by heating before loading onto the gel (Lee, Zo, Kim, 1996). A shortcoming of this method is that some of the fragments re-anneal, causing up to three bands per sequence to appear on the gels (one for each single strand and one for the re-annealed double strand). This problem can be overcome by using one phosphorylated primer and digesting the phosphorylated strands with lambda exonuclease (Schwieger, Tebbe, 1998). Scheinhert *et al.*, (1996) also circumvented

the problem of re-annealing double strands by performing magnetic separation of fragments prior to electrophoresis, after amplification of DNA with one biotinylated primer. Electrophoretic conditions such as temperature and gel polymerization can heavily influence DNA fragment separation and must be standardised to ensure precise results. The identity of bacterial phylotypes can be obtained by excising and sequencing SSCP gel bands and SSCP gels can be hybridized with specific probes.

Randomly Amplified Polymorphic DNA (RAPD)

RAPD employs random, short oligonucleotide primers to PCR amplify community DNA. Because the primers are short random sequences, PCR-generated DNA fragments will be of differing lengths and changes in the template DNA (brought about by changes in the community) will be reflected as different patterns of PCR fragments separated on agarose gels. Although the technique is seemingly simple and can be automated, it suffers from several drawbacks (Muyzer, 1999). RAPD is very sensitive to variations in PCR conditions (template DNA quality, MgCl₂, and primers), banding patterns from very complex communities (e.g. soil communities) may be overly complex to analyse and no phylogenetic information is available.

Restriction Fragment Length Polymorphism (RFLP)

RFLP analysis of microbial communities involves digesting PCR amplified community DNA (usually 16S rDNA) with restriction enzymes, electrophoresing the restriction products and monitoring the banding patterns on the gels (Massol-Deya, Odelson, Hickey, Tiedje, 1995). Although the method is straightforward and does not require any expensive equipment it does have several shortcomings. The number of bands does not reflect the number of phylotypes present in the sample under analysis and no phylogenetic information is obtained.

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

To a certain extent, the shortcomings of RFLP are overcome by T-RFLP, which utilises fluorescently labelled primers in the initial PCR. The products are then digested and analysed on an automated sequencer, which detects only the labelled fragments (terminal fragments). T-RFLP is very sensitive, has been used to analyse shifts in very complex communities (Liu, Marsh, Cheng, Forney, 1997; Ludemann, Arth, Liesack, 2000; Lueders, Friedrich, 2000) and allows for band quantification, but

prohibits band hybridization or sequencing, requires expensive fluorescent primers and requires access to an automated sequencer (Muyzer, 1999).

1.3.1.2 Methods utilised for assessment of microbial community diversity in this study

The study undertaken required assessment of many samples over a two-year period, so a fingerprinting technique was chosen to assess and compare genetic diversity in sediment microbial communities after alterations to environmental conditions. Of the fingerprinting techniques described above all are able to do this quickly and efficiently, but only DGGE and SSCP are able to provide phylogenetic information about the community by sequencing or hybridization. For this reason, and because SSCP has problems with reproducibility (Muyzer, 1999), DGGE was utilised to fingerprint the microbial communities of sediments in this study.

DGGE, like any technique used in microbial ecology, has its limitations. These limitations need to be understood in order to utilise the technique and interpret the results successfully. The very premise of DGGE is the separation of DNA fragments such that one band equals one operational taxonomic unit (OTU). That is, that each phylotype will produce only one band and that each band will represent only one phylotype. Neither of these statements is necessarily true in regard to DGGE. Some strains of bacteria possess more than one 16S rRNA operon and therefore produce multiple bands on DGGE gels (Nubel, Engelen, Felske, Snaird, Wieshuber, Amann, Ludwig, Backhaus, 1996). This should not present a problem to band pattern interpretation, but may make banding patterns more complex, or even uninterpretable in very complex communities. Care needs to be taken though, when trying to use band number to infer the number of phylotypes present. It may be said that the number of bands present represents 16S rRNA gene diversity, but not necessarily bacterial strain diversity. It has also been noted that DNA fragments of different sequence may have identical mobilities. This problem can be overcome to a certain extent by excising and sequencing several bands of the same mobility to ensure they are indeed the same sequence. This is, though, not practical when very complex communities are analysed. For this reason it is assumed that each band is a different

OTU, even though this assumption may overestimate or underestimate the true diversity. The sequence information gained from DGGE can be limited because the size of the fragment used in the analysis is relatively short. DGGE fragments are short (up to 500 bp) because separation of longer fragments is reduced (Heuer, Smalla, 1997). These short sequences can though, be used to gain valuable information on the makeup of the microbial community and to monitor the efficacy of the whole DGGE technique (Bowman, McCammon, Gibson, Robertson, Nichols, 2003). The limited sequence information obtained does not affect the performance of DGGE in its primary task; that of allowing enough samples to be processed to meaningfully monitor changes in microbial communities. It does, though, provide a useful means to monitor the uniqueness of bands and give insights into community composition that would otherwise not be obtained.

Finally, the limitations of community resolution inherent in DGGE should also be considered. Fingerprint techniques, such as DGGE, are said to represent the most abundant 1% of the community at most (Muyzer, de Waal, Uitterlinden, 1993). This low threshold detection may limit the ability of DGGE to elucidate changes in community richness, especially when using universal primers. Although the detection limit in DGGE is often seen as a negative, this is not necessarily so. Sediment communities are extremely diverse and the sampling effort needed to reliably sample such communities is immense (Curtis, Sloan, 2004). If only small (even though they may be considerable in terms of time and expense) surveys are carried out, then communities may appear similar, even though they exhibit a difference when only the most abundant species are considered. Bowman *et al.* (in preparation), experienced this very phenomenon. 16S rDNA clone library surveys of the reference sediments used in my study showed the communities to be not significantly different, despite having different physical and chemical attributes. DGGE analysis of the same communities, though, indicated that they were different. This apparent inconsistency in results suggests that the communities were highly diverse, that a large sampling effort is required to reliably sample the community (greater than the 350+ clones sampled for each sediment) and that to monitor community changes less resolution (to elucidate major changes) or more sampling effort was required. DGGE provides such a technique; providing the ability to analyse statistically meaningful numbers of samples to detect changes to the most abundant members of microbial communities.

The range (resolution) of the technique can be further extended by utilising primers targeting less dominant, but ecologically important, community members (e.g. ammonia oxidising bacteria).

Clone libraries were also utilised in this study to complement DGGE and allow a more comprehensive assessment of microbial diversity in these sediments. The clone library data will complement the sequence information obtained from DGGE and allow an assessment of community diversity that DGGE would not, but is not suited for the routine monitoring that was undertaken as the major part of this study.

1.4 Experimental design

This study was undertaken as part of an Aquafin CRC study into the effects of salmon-farming on the Tasmanian marine environment (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). This study examined the effect of organic carbon deposition on sediments under salmon cages during periods of farming and fallowing. The point source nature of the organic loading under salmon cages, and the controlled nature of the loading and its cessation, presented an ideal opportunity to study changes in sediment microbial communities after anthropogenic perturbation. The following chapter largely paraphrases chapter 2 of the report generated from the CRC study. Farm production data are taken from sections 4.2 and 5.2 of the CRC study report.

1.4.1 Site Locations

Two Salmon farms, exhibiting different environmental conditions, were chosen for this study. The farms were located on the Tasman Peninsula (lat: 43°6' S, Longitude: 147°45' E) (Farm 1) and in the D'Entrecasteaux channel (Latitude: 43°19' S, Longitude: 147°1' E) (Farm 2), Tasmania, Australia (Figure 1- 1). At farm 1, depth ranged from 15 -20m. Farm 1 was a completely marine environment, exposed to westerly and south-westerly winds and was also heavily influenced by wave and swell action. As a result sediments at farm 1 were predominantly fine sands, with a low percentage of silt-clays. Farm 2 was situated in deeper water; 35-40m. Farm 2 was also primarily marine in nature, although periods of large freshwater run-off did

occur. The lease was exposed to the northwest and protected from most wave action and ocean swells. As a result, sediments were predominantly silt-clays.

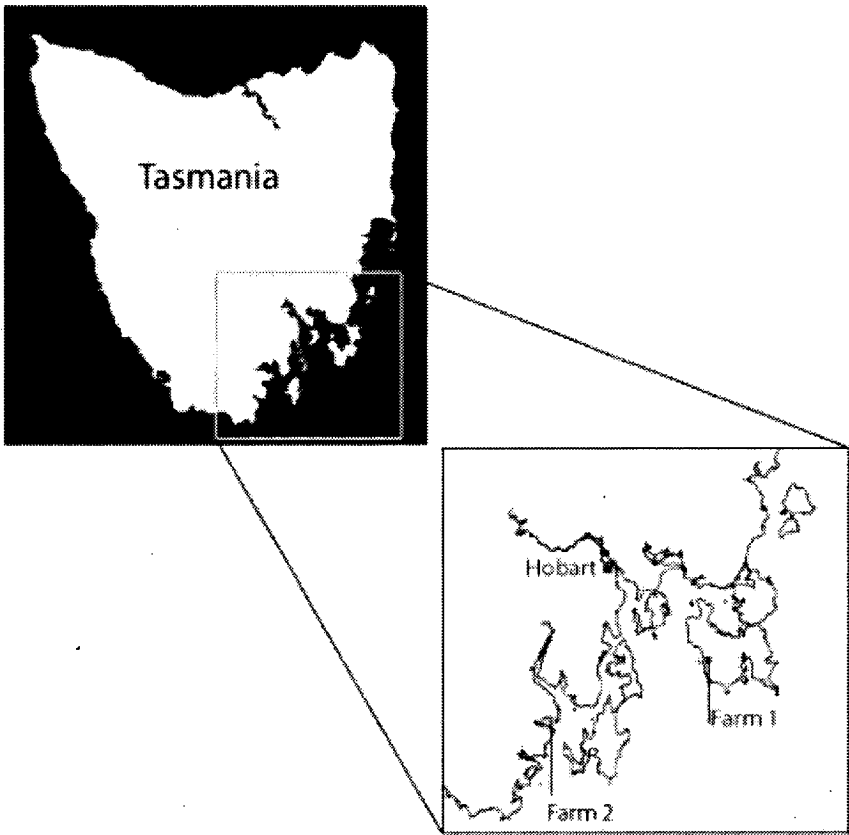


Figure 1-1 Map of Tasmania, Australia detailing study site locations (inset). After (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004)

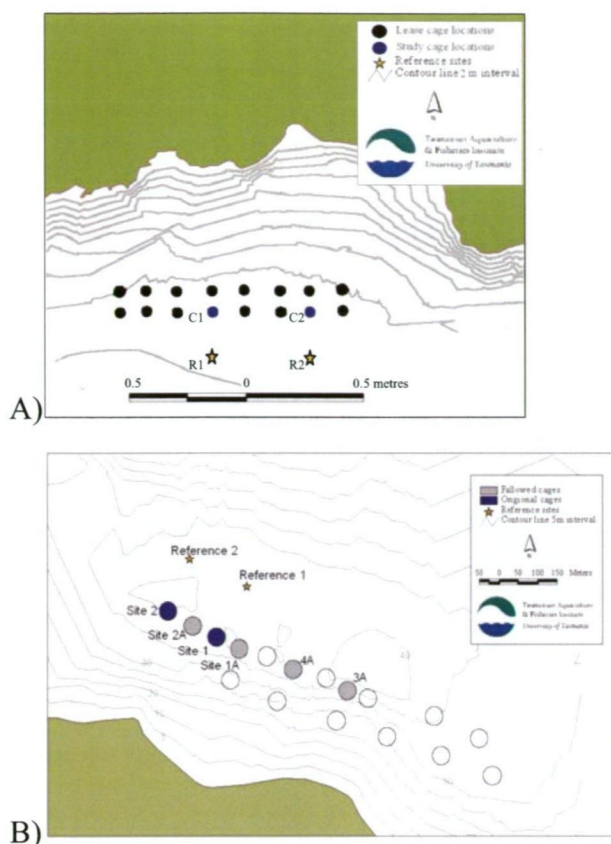


Figure 1-2. Lease location and cage and reference site location at A) Farm 1 (2m contour intervals) and B) Farm 2 (5m contour intervals). Taken from (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004)

1.4.2 Farm production and history details

1.4.2.1 Farm 1

Farm 1 was first stocked with Atlantic Salmon in May, 1995. Between this time and 2000 the lease was stocked for at least eight months per year. When not stocked the whole lease was fallow, for periods of 1-4 months. Farm production increased from 394 tonnes in 1998, to 1336 tonnes in 2001. The farm 1 lease area and cage layout are shown in Figure 1- 2a. Cage 1 was initially stocked for five months in 1997 and three months in 1999. It was then stocked again in mid-2000 and given a two month fallow period before the commencement of this trial. Prior to this trial cage 2 was stocked for the second halves of 1997 and 2000 and subsequently fallowed for 2.5 months. The fallowing policy at farm 1 is such that all cage positions are fallowed during the year, but on a rotational basis. At no time in the study was the entire lease fallow.

Average fish weight at farm 1 remained constant over both production cycles, but biomass and feed input were reduced during the second production cycle (Figure 1-3). Daily feed rates were highly variable (Figure 1-4); primarily a result of difficult and variable weather conditions.

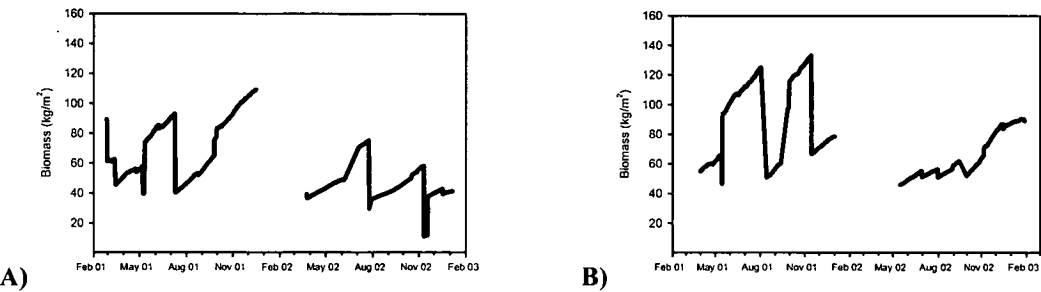


Figure 1-3 . Cage biomass for study cages at Farm 1 during both production cycles. A) Cage 1 and B) Cage 2. Taken from (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004).

Cage sites experienced different length fallowing periods at the end of the first production cycle as a result of farm management difficulties. Cage 1 received three months fallowing, whilst cage 2 was fallow for an additional six weeks. Consequently, the two positions were not treated as replicates in the second production cycle, but were considered separately with a single reference site to give the following pairings: cage 1/reference 1 and cage 2/reference 2.

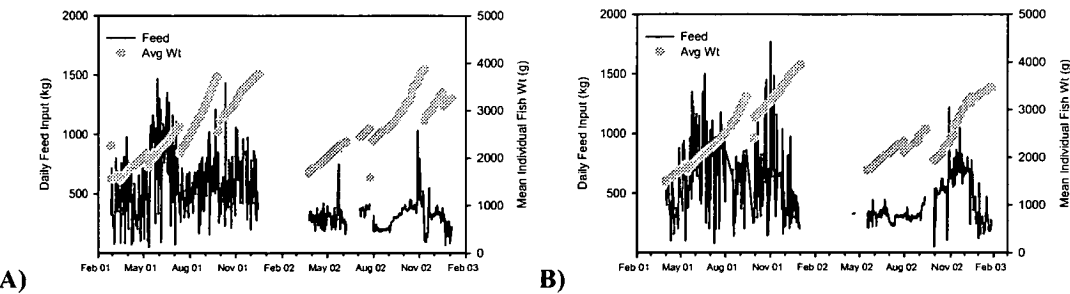


Figure 1-4 Farm production information for Farm 1 including mean individual fish weight and daily feed input at study cages over both production cycles. A) Cage 1 and B) Cage 2. Taken from (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004).

1.4.2.2 Farm 2

Between 1989 and 1999, farm 2 was stocked at varying intensities and had a number of position changes and expansions. Cages 1, 2, 1A and 2A are all in the area granted as a lease extension in 1999, and had not previously been farmed. The position and size of the farm 2 lease and study cages are shown in Figure 1- 2. Biomass and mean fish weight were similar in cages studied at Farm 2 over both production cycles (Figure 1- 5) and were broadly equivalent to the levels at Farm 1 in the second production cycle. Daily feed input did, however, decline during the second production cycle (Figure 1- 6).

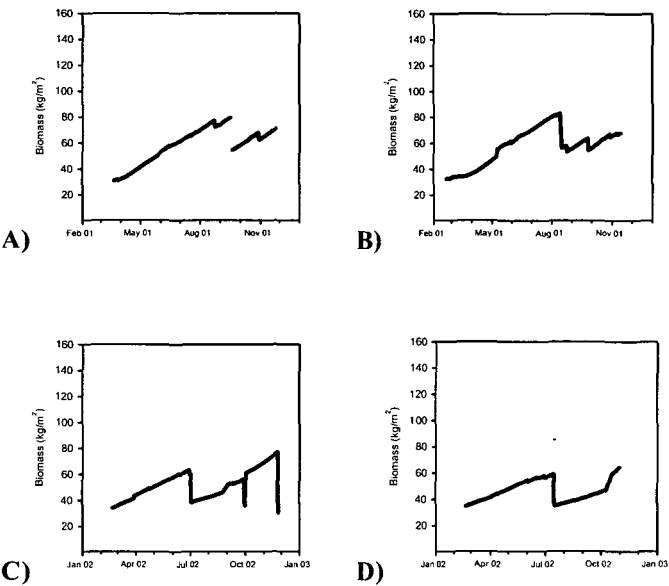


Figure 1-5 Cage biomass for study cages at Farm 2 during production cycle 1 - (A) Position 1 and (B) Position 2; and production cycle 2 - (C) Position 1A, (D) Position 2A. Taken from (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004)

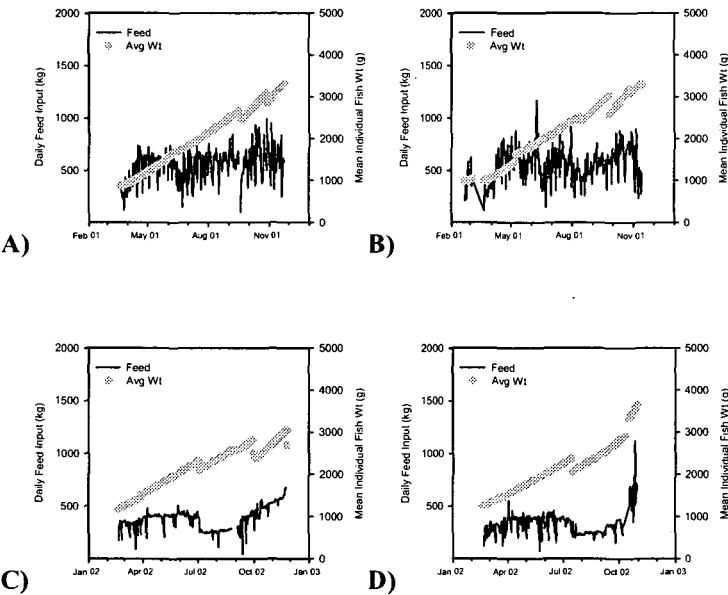


Figure 1-6 Farm production information for Farm 2 including mean individual fish weight and daily feed input at study cages during production cycle 1 - (A) Position 1 and (B) Position 2; and production cycle 2 - (C) Position 1A, (D) Position 2A. Taken from (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004).

1.4.3 Basic sampling design

At both farms, two cages (cage 1 and 2) were randomly selected from within the leases (Figure 1- 2). Two reference sites were also selected at each farm, 150m from the edge of each study cage. Reference sites exhibited the same depth and particle size distribution as their respective cages. Sites were sampled at the beginning of each production cycle, after nine months stocking, and after 3 months fallowing. The second production cycle commenced immediately following the completion of the fallowing period. At farm 1, however, management was unable to adhere to this schedule at one of the cages. At cage 2 the first fallow period continued for 4.5 months, six weeks longer than anticipated. After this, cage 2 reverted to the anticipated 9 month stocking, 3 month fallow production schedule. Because cage 2 was 6 weeks behind cage 1 in the second cycle, the two cages could no longer function as treatment replicates. For this reason during the second cycle at farm 1 all analysis was completed on cage 1/Reference 1 and Cage 2/Reference 2, separately

1.4.4 Sample collection and preparation

Triplicate sediment cores were collected from each cage and reference site for further analysis. Cores were collected using polyethylene tubes (150 mm diameter) and a Craib corer. Cores were all at least 100 mm deep. After collection, cores were stored in an ice cooler filled with ambient water until transfer to the laboratory. Cores were then sliced at three depths (0-2 mm, 2-5 mm and 5-10 mm). Samples for microscopy were then fixed in 4% formalin in 0.2 µm filtered seawater and stored at 4°C until bacterial dispersions for enumeration were performed. For molecular analysis, all depth profiles were combined (to give sediment to 1cm), mixed well and stored at -20°C until DNA extraction could be performed.

Chapter Two

Bacterial enumeration in salmon farm sediments

Chapter 2 Bacterial Enumeration in Salmon farm sediments

2.1 Introduction

Bacteria exhibit an immense range of diversity, express a wide variety of metabolic activities and are able to survive extreme environments. They play a major role in biogeochemical cycling and are often present in high numbers. Bacteria are able to metabolize organic matter and convert it to biomass through bacterial growth, making it ultimately available to higher trophic levels (Gregori, Citterio, Ghiani, Labra, Sgorbati, Brown, Denis, 2001). The presence of bacteria in marine environments may also affect human health and water quality. Pathogenic bacteria may be present both in the water column and in the sediment, and may impact directly on human health or on the health of aquatic live-stock. Estimates of bacterial abundance, biomass and community structure are paramount to assessing the roles of bacteria in food webs and biogeochemical cycling, and to understanding bacterial population dynamics in natural systems (Buesing, Gessner, 2002).

Coastal areas are highly productive, commonly receive large amounts of anthropogenic organic matter, and are important in the global cycling of organic matter. The fate of organic matter in near-shore areas may be summarized thus: particulate organic matter may be trapped in the immediate area, biologically utilized by heterotrophic organisms in the water column or sediments or exported from the local area to the open ocean (Zimmerman, Canuel, 2001).

It has long been thought that organic matter decomposition and bacterial growth are highly dependant on the presence of oxygen. Anoxic conditions were thought to result in slow mineralization rates and bacterial growth (Bastviken, Ejlertsson, Tranvik, 2001; Hedges, Hu, DEvol, Hartnett, Tsamakis, Keil, 1999; Simon, Poulecek, Velimirov, Mackenzie, 1994). However other studies (Blume, Bischoff, Reichert, Moorman, Konopka, Turco, 2002; Rysgaard, Thamdrup, Risgaardpetersen, Fossing, Berg, Bondo Christensen, Dalsgaard, 1998) suggest that the lability of organic matter determines its susceptibility to degradation. These studies suggest that redox has little effect on the mineralization rates of labile organic matter, but that refractory organic matter is more effectively degraded under oxic conditions. It would appear then that apparent differences in overall degradation rates may be due to the lability of organic

matter, rather than the actual pathways involved in its decay. Higher degradation rates in the surface, oxic zones may reflect the fact that these zones are the first to encounter organic material, not necessarily that they are more efficient in its degradation (Kristensen, 1988). In areas of high sedimentation and mineralization, rates of anaerobic decomposition and bacterial growth may be higher than those in oxic zones.

Marine sediment bacterial communities exhibit large amounts of heterogeneity, both horizontally and vertically (Bowman, McCuaig, 2003; Wilde, Plante, 2002). This spatial heterogeneity has been found to manifest itself as highest activity and abundance at the surface and decreasing with depth (Luna, Manini, Danovaro, 2002; Novitsky, 1983a; b). It has also been observed (Freitag, Klenke, Krumbein, Gerdes, Prosser, 2003; Sundback, Jonsson, Nilsson, Lindstrom, 1994) that as sediment conditions become more extreme, under euxenic conditions for example, bacterial growth may also become retarded.

In the present study, microbial numbers, biomass and cellular morphological characteristics were assessed in sediments with high point-source organic loading (under two fish-farms) and at reference sites with similar sediment characteristics, but without the organic loading. These parameters were assessed during farming and after a fallowing period, over two full production cycles, to investigate the effects of farm management practices on the microbial community. Assessments were also carried out over a vertical profile to determine the importance of labile organic matter and oxygen in maintaining/promoting microbial biomass.

2.2. Materials and Methods

2.2.1 Bacterial enumeration

Sediment core collection is described in chapter 1.4.4. Cores for bacterial counts were sliced at three depths (0-2 mm, 2-5 mm and 5-10 mm). Samples were then fixed in 4% formalin in 0.2 μm filtered seawater and stored at 4°C until bacterial dispersions were performed. Bacterial dispersions were carried out after Epstein *et al.* (1995). Samples were sonicated (Misonix, “microson XL”) four times for 20s, with at

least 30s on ice between sonications. Samples were then washed four times and the supernatants pooled. An appropriate dilution was then chosen to ensure countable cell densities prior to staining.

2.2.2 Cell staining and microscope slide preparation

Bacterial cells were stained after the method of Noble and Fuhrman (1998). Bacterial suspensions were stained, in the dark, for 20 min. with SYBR Green I nucleic acid stain (Molecular Probes). A 25 mm glass filter holder (Millipore) was used to filter the stained sample through a 0.02 μm pore size Al_2O_3 Anodisc 25 membrane filter (Whatman), backed by a 0.8 μm cellulose mixed ester membrane filter (Millipore type AA). The Anodisc filter was mounted on a glass slide with a drop of antifade (50% glycerol, 50% phosphate buffered saline (0.05 M Na_2HPO_4 , 0.85% NaCl, pH 7.5) with 0.1% *p*-phenylenediamine). Ten fields of view were randomly selected, and at least 300 cells counted by image analysis, on a Leica DMRBE microscope with 100x objective (Leica PL Fluotar), under blue excitation. Samples were analysed blind to reduce operator bias (Gough, Stahl, 2003). A sub sample of filters were also counted manually to confirm the accuracy of automated counts. Images were acquired with a Leica DC 300F charged-coupled device (CCD) camera using Leica IM50 software. All pictures were recorded as 8-bit images of 1300 x 1030 pixels.

2.2.3 Image Analysis

Image analysis was performed using Reindeer graphics' Fovea Pro and Adobe Photoshop 7.0 software by employing the following steps. Images were converted to grey-scale and subjected to a Laplacian 5X5 filter to enhance cell boundaries. A Gaussian 5X5 filter was then applied to remove any noise created by the Laplacian filter. Images were then thresholded as described by Viles and Sieracki (1992) using the global visual threshold. Cells were then counted and cell parameters measured. All counts are presented as cells/g sediment (wet weight).

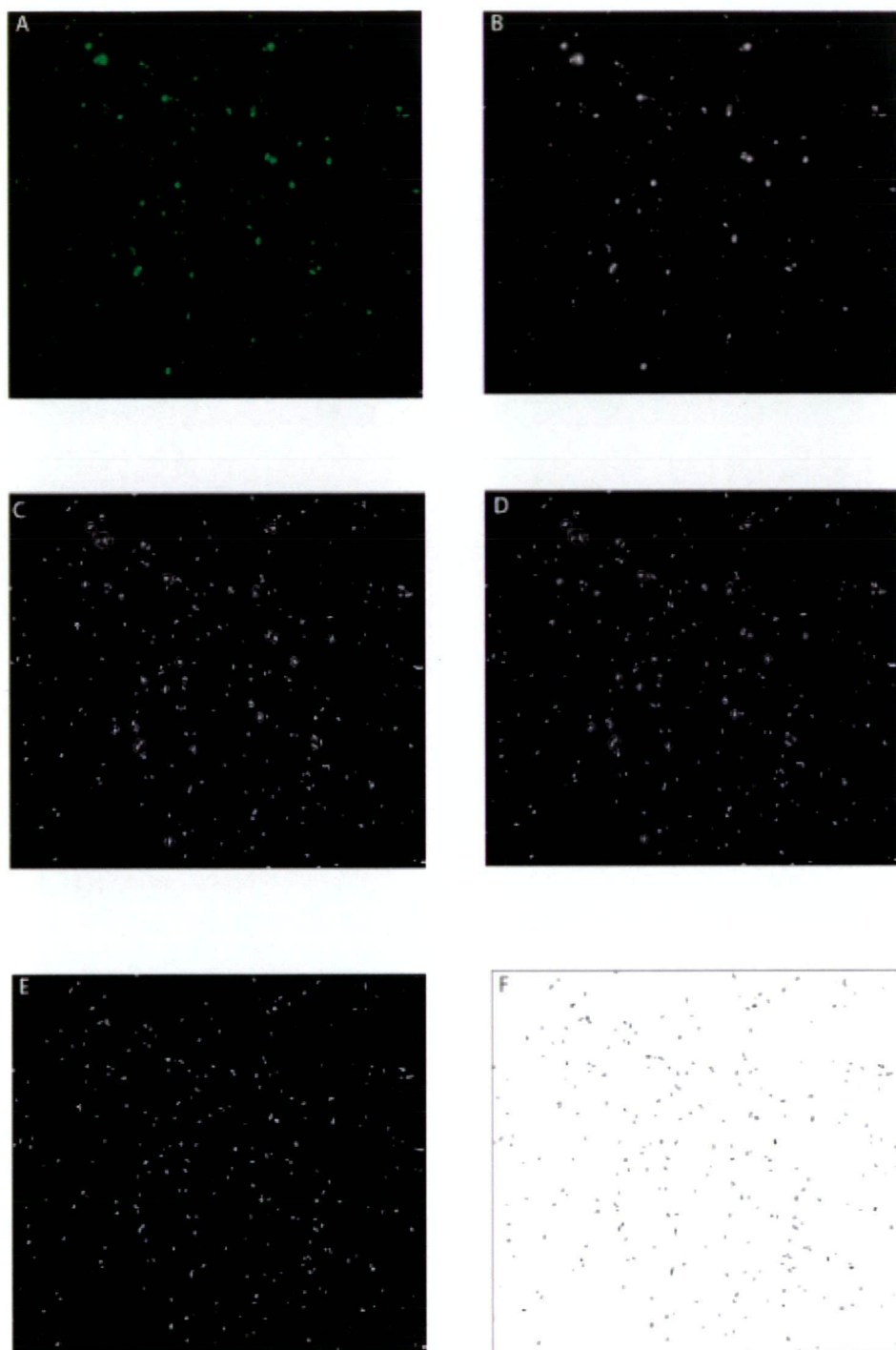


Figure 2-1. Steps involved in image analysis data acquisition. A) Raw SYBR green image, B) Gray scale conversion of raw image, C) Image sharpened with 5X5 Laplacian filter, D) Gaussian smoothed image, E) Thresholded image and F) Final inverted thresholded image ready for data acquisition.

2.2.4 Determination of bacterial cell volume and biomass

Cell volume was determined according to Loferer-Krossbacher *et al.* (1998) using cell measurements obtained by image analysis. Bacterial biovolume was then converted to carbon content, assuming $310 \text{ fgC } \mu\text{m}^{-3}$ (Fry, 1990).

2.2.5 Statistical Analysis

Analysis of variance (ANOVA) was used to test for the effect of treatment (2 levels: farm site and reference site), time (3 or 4 levels: times throughout the stocking period) and sediment depth (3 levels: 0-2 mm, 2-5 mm, 5-10 mm) on microbial numbers, microbial biomass and cellular morphology. Samples were obtained as explained in section 1.4.3 (Basic sampling design), and data were analysed with this sampling design in mind. Homogeneity of variances were checked visually by examining residual plots. Data that did not meet this assumption of ANOVA were log transformed. Significant factors were then compared using Tukey's HSD post hoc tests or *a priori* planned comparisons between each cage and reference site at each sampling time, but not between sampling times. When non-orthogonal planned comparisons were made, a Bon-Feroni correction was used to adjust α . All statistical tests were performed at $\alpha = 0.05$ using the statistical software SPSS v10.

2.3 Results

Generally, the total count method employed was successful in enumerating sediment bacteria, although it was not possible to count the very large filamentous bacterial types (e.g. *Beggiatoa* spp.) that were observed in wet mounts of the sediments (Figure 2- 1). The sonication process employed to disperse the bacterial cells from the sediment particles may have been too harsh for these large bacteria. Although beyond the scope of this study, *Beggiatoa* filaments were counted in these sediments as part of the larger FRDC study that this study complemented (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004).

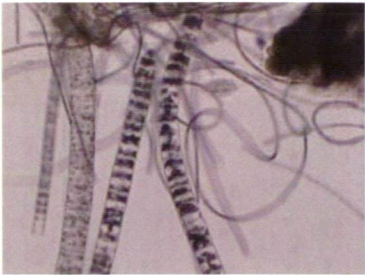


Figure 2-2 Wet mount showing *Beggiatoa* spp. observed in cage sediments. Photo courtesy C.Burke.

2.3.1 Farm 1

2.3.1.1 Bacterial counts over farm cycle one

Mean microbial direct counts ranged from $1.56 \times 10^8 \pm 6.1 \times 10^7$ to $4.78 \pm 1.1 \times 10^8$ cells/g sediment at reference sites and from $5.39 \pm 2.7 \times 10^8$ to $3.0 \pm 1.0 \times 10^9$ cells/g sediment at cage sites. Results of the three-way factorial ANOVA performed are presented in Table 2- 1. A significant interaction was detected in the effects of treatment and time on microbial numbers.

Table 2-1 ANOVA table for log mean counts (cells/g) at farm 1 during the first 12 month stocking period (April 2001 – March 2002).

Source	df	Mean Square	F	Sig. (p)
Treatment	1	11.377	444.4	0.000
Time	2	2.610	101.954	0.000
Depth	2	0.168	6.559	0.002
Treatment x Time	2	0.425	16.620	0.000
Treatment x Depth	2	0.0409	1.599	0.209
Time x Depth	4	0.0213	0.786	0.537
Treatment x time x depth	4	0.0225	0.879	0.480
error	78	0.0256		
Total	96			

Microbial numbers were significantly higher at the cage sites than at reference sites at each sampling time ($p<0.05$). Microbial numbers increased with organic loading

from the farm over the nine month stocking period and decreased during the three month fallowing period (Figure 2- 2A). Although bacterial numbers continued to rise between 4.5 and nine months stocking, no significant difference was observed between these times. The large standard error observed at nine months for the cage site may have resulted from different stocking and feeding rates at each site (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). Fish at one replicate cage were harvested part-way through the stocking period and not restocked for several weeks. As a result organic loading for this replicate was not as high or sustained as that received by the other replicate cage.

ANOVA showed a significant effect of sediment depth on bacterial numbers (Table 2- 1). Bacterial counts were higher in the surface layer than they were in the deepest layer irrespective of treatment (Table 2- 2).

Table 2-2 Mean bacterial counts (cells/g sediment) \pm standard errors at each depth over the two year trial. Means sharing a common superscript within each stocking cycle (year) are not significantly different ($p>0.05$).

DEPTH	Year 1 (n=44)	Year 2; Cage 1, Ref 1 (n=32)	Year 2; Cage 2, Ref 2 (n=24)
0-2 mm	$1.25 \times 10^9 \pm 2.6 \times 10^8$ <i>a</i>	$8.87 \times 10^8 \pm 1.9 \times 10^8$ <i>a</i>	$6.20 \times 10^8 \pm 1.4 \times 10^8$
2-5 mm	$1.11 \times 10^9 \pm 2.9 \times 10^8$ <i>b</i>	$6.62 \times 10^8 \pm 1.3 \times 10^8$ <i>ab</i>	$5.00 \times 10^8 \pm 1.2 \times 10^8$
5-10 mm	$8.25 \times 10^9 \pm 1.5 \times 10^8$ <i>b</i>	$6.16 \times 10^8 \pm 1.3 \times 10^8$ <i>b</i>	$4.20 \times 10^8 \pm 8.4 \times 10^7$

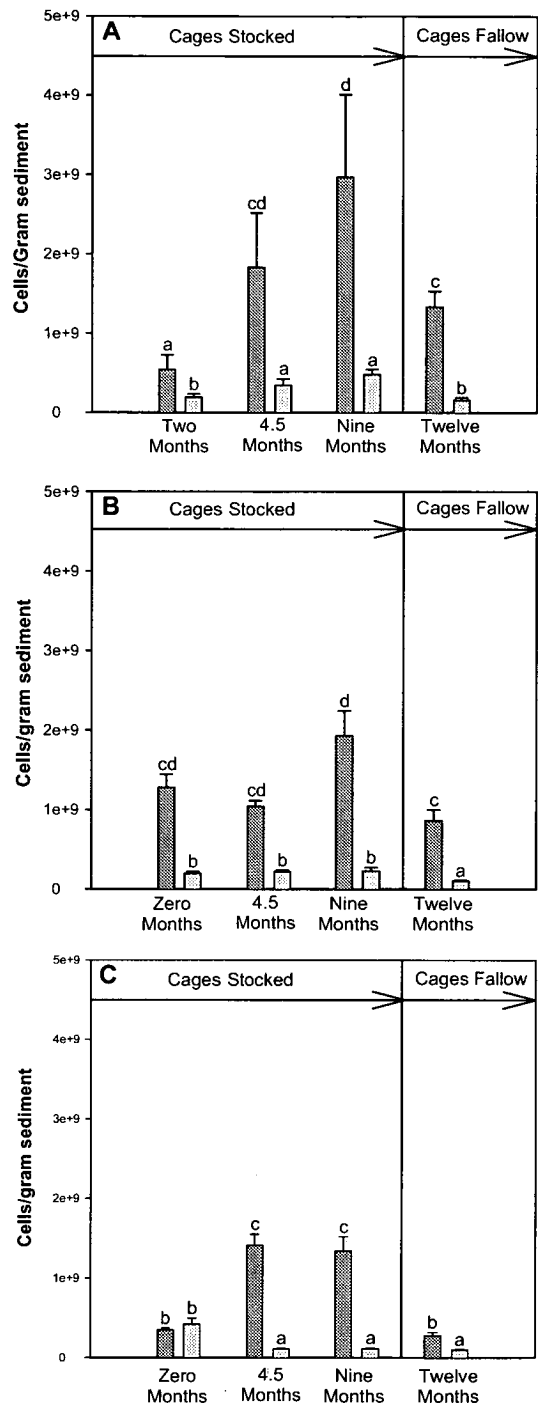


Figure 2-3 Mean bacterial numbers (cells/g sediment) at farm 1 over the two, 12-month farm cycles. **A)** First 12 month cycle (n=4 at two months, n=6 at remaining sampling times). **B)** Cage 1 and Reference 1 during the second 12 month cycle (n=3). **C)** Cage 2 and Reference 2 during the second 12 month cycle. Months indicate time through the respective farm cycles. Superscripts refer to Tukey’s post hoc tests of all treatment/time combinations within each graph. Bars sharing a common superscript are not significantly different (p>0.05). Bar ± standard error. Red bars indicate cage sites, green bars indicate reference sites.

2.3.1.2 Bacterial counts over farm cycle two

During the second 12 month cycle mean bacterial numbers ranged from $8.6 \pm 1.4 \times 10^8$ to $1.9 \times 10^9 \pm 3.1 \times 10^8$ cells/g sediment at cage 1, and from $2.3 \times 10^8 \pm 1.5 \times 10^7$ to $1.0 \times 10^8 \pm 7.5 \times 10^6$ cells/g sediment at reference 1. ANOVA results are presented in Table 2- 3. Bacterial numbers were higher at the cage site than the reference site at the beginning of the stocking period, and remained higher for the entire 12 month period (Figure 2- 2B). Bacterial numbers at the cage site did not increase significantly over the nine month stocking period, but did decline over the fallow period to become significantly less than seen at the end of the stocking period, but not different from the start of the cycle. Bacterial numbers were higher in the surface sediments (Table 2- 2).

Table 2-3 ANOVA table for log mean counts (cells/g) at farm 1, cage 1, reference 1 during the second 12 month stocking period (March 2002 – March 2003).

Source	df	Mean Square	F	Sig. (p)
Treatment	1	12.517	498.8	0.000
Time	3	0.344	13.706	0.000
Depth	2	0.101	4.013	0.024
Treatment x Time	3	0.0568	2.266	0.093
Treatment x Depth	2	0.0124	0.494	0.613
Time x Depth	6	0.065	0.259	0.953
Treatment x time x depth	6	0.0536	2.136	0.066
error	48	0.0251		
Total	72			

During the second cycle, Cage 2 was left fallow for 4.5 months, instead of three months. ANOVA results assessing the effects of farm loading on bacterial numbers at cage 2 and reference site 2 during the second 12 month farm cycle are presented in Table 2- 4. This extra fallow period allowed bacterial numbers to return to reference levels at the start of the new stocking period (Figure 2- 2C). Microbial numbers increased rapidly during the stocking period, and declined during the fallow period. Although they did not return to the same levels as the reference site, they were at a

level not significantly different from the pre-stocking level at both cage and reference sites. Although there was no statistically significant effect of depth on bacterial numbers at cage 2/reference 2 (Table 2- 4) during the second 12 month farm cycle, a trend of higher numbers in the sediment surface can be seen (Table 2- 2).

Table 2-4 ANOVA table for log mean counts (cells/g) at farm 1, cage 2, reference 2 during the second 12 month stocking period (April 2002 – April 2003).

Source	df	Mean Square	F	Sig.
Treatment	1	7.348	411	0.895
Time	3	0.646	36.119	0.693
Depth	2	0.132	7.376	0.235
Treatment x Time	3	1.402	78.435	0.831
Treatment x Depth	2	0.0068	0.382	0.016
Time x Depth	6	0.0151	0.844	0.095
Treatment x time x depth	6	0.0109	0.612	0.071
error	48	0.0179		
Total	72			

2.3.1.3 Additivity of organic load on bacterial numbers

To determine the additivity of farm effects on bacterial numbers, cage sites were compared at the beginning of the trial (two months), at the end of the first fallowing period (12 months for cage 1, 12 and 13.5 months for cage 2) and at the completion of the trial (end of the second fallowing period: 24 months). Because cages were not treated in the same manner during the first fallowing period and second cycle the comparison was made for both cage sites separately.

For cage 1 there was a significant effect of time on the bacterial numbers ($F_{2,21} = 4.483$, $p < 0.05$). After the first 12 month cycle, bacterial numbers were significantly higher than at the beginning of the trial. After 24 months, bacterial numbers had shifted towards an intermediate count (Figure 2- 3A).

A similar pattern is evident for Cage 2 ($F_{3,29}=49.81$, $P<0.001$), but the additional fallowing time given cage 2 resulted in a further decline in microbial numbers. Bacterial numbers were elevated above those at the beginning of the trial after 12 months (the completion of the initial three month fallowing period), but declined during the 1.5 month additional fallowing period to levels not significantly different from those at the beginning of the trial. After the second cycle bacterial numbers had actually significantly declined to below those detected at the beginning of the trial (Figure 2-3B).

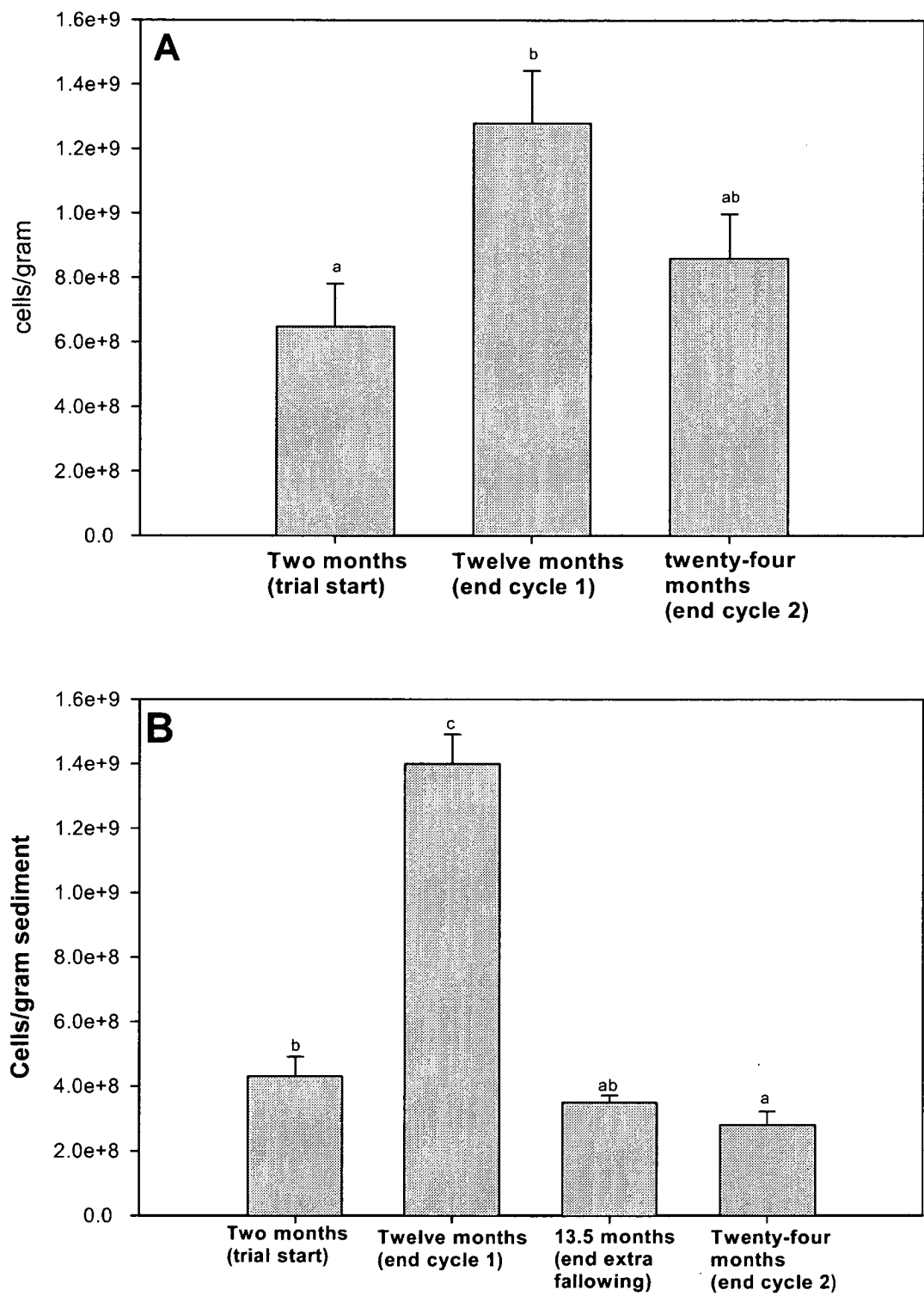


Figure 2-4 Mean bacterial numbers (cells/g sediment) at farm 1 cage sites (cage 1 (A) and cage 2 (B)) during the twenty-four month trial (n=3). Bars within each graph sharing a common superscript are not significantly different ($p>0.05$). Bar \pm standard error)

2.3.1.4 Cellular morphology over farm cycle one

ANOVA results for cell area (tested over the same factors as above) are presented in Table 2- 5.

Table 2-5 ANOVA table for mean cell size (μm^2) at farm 1 during the first 12 month stocking period (April 2001 – March 2002).

Source	df	Mean Square	F	Sig. (p)
Treatment	1	9.692×10^{-4}	0.198	0.661
Time	2	0.0327	6.657	0.005
Depth	2	0.0023	0.455	0.640
Treatment x Time	2	0.0266	5.426	0.011
Treatment x Depth	2	6.397×10^{-4}	0.130	0.878
Time x Depth	4	0.0019	0.393	0.811
Treatment x time x depth	4	0.0038	0.689	0.607
error	24	0.00491		
Total	42			

Mean cell size at cages was significantly different from that at reference sites at each sampling time, although not always in the same direction (Figure 2- 4A). Cell size was initially smaller at the cage sites than at reference sites, but increased with time to be larger by the end of the first 12 month sampling period. Planned comparisons between each cage site at each time showed they were significantly different from one another at each sampling ($p < 0.05$), while comparisons between the reference sites showed no significant change in cell size over time ($p > 0.05$) (superscripts not shown).

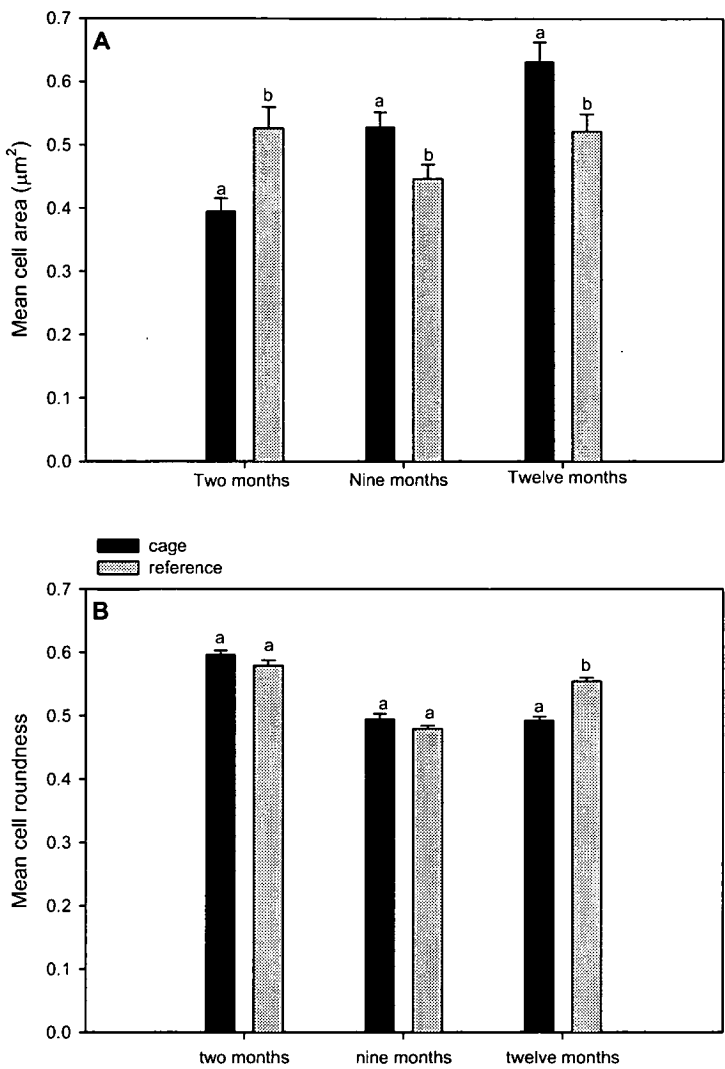


Figure 2-5 Mean size (μm^2) (A) and roundness (B) of bacterial cells in sediments from farm 1 over the first 12 month farm cycle. Superscripts refer to planned comparisons between cage and reference sites at each sampling time, but not between sampling times. Bars sharing a common superscript are not significantly different ($p>0.05$). Bar \pm standard error.

Cell morphology was measured as “roundness” ($4\text{Area}/\pi\text{Length}^2$). Again a 3-way ANOVA showed no significant interaction between sediment depth, treatment and time; depth and time or depth and treatment. There was however a significant interaction between treatment and time, but no significant effect of depth on roundness (Table 2- 6).

Table 2-6 ANOVA table for mean cell roundness at farm 1 during the first 12 month stocking period (April 2001 – March 2002).

Source	df	Mean Square	F	Sig.
Treatment	1	0.0045	8.185	0.009
Time	2	0.0268	48.881	0.000
Depth	2	1.050 X 10 ⁻⁵	0.019	0.981
Treatment x Time	2	0.00583	10.362	0.000
Treatment x Depth	2	5.95 X 10 ⁻⁴	1.084	0.354
Time x Depth	4	2.89 X 10 ⁻⁴	0.526	0.718
Treatment x time x depth	4	1.92 X 10 ⁻⁴	0.350	0.842
error	24	5.49 X 10 ⁻⁴		
Total	42			

Cells at the reference sites were significantly more round than those at the cage sites at the 12 month sampling time ($p<0.05$), but were the same shape as those at the cage sites at the two and nine month sampling times ($p>0.05$). Cells became more rod shaped (less round) at both cage and reference sites for the first nine months, after which they became more round at the reference site, but remained elongated at the cage site (Figure 2- 4B). This change in roundness was similar to the trend shown in cell size at the two sites.

2.3.1.5 Cellular morphology at Cage 1 and Reference 1 over farm cycle two

A 3-way factorial ANOVA (factors as above) indicated no significant interactions, but significant effects of time ($F_{3,48}=25.79, p<0.000$) and treatment ($F_{1,48}=6.05, p=0.018$) on cell size (area) at cage 1 and reference 1. Cell size decreased over the nine month stocking period and increased over the fallowing period. Cell size did not, however, return to the pre-stocking size (Figure 2- 5A). Cell size was always significantly larger at the cage sites ($p<0.05$).

Table 2- 7 shows ANOVA results for the effects of treatment, time and depth on cell shape. There was a significant interaction between treatment and time (Figure 2- 5B).

Cell shape remained constant at reference 1 until the 12 month sampling, when it changed significantly to become more elongated. At the cage site, cell shape gradually changed to become significantly more round as the sampling period progressed (Figure 2- 5B).

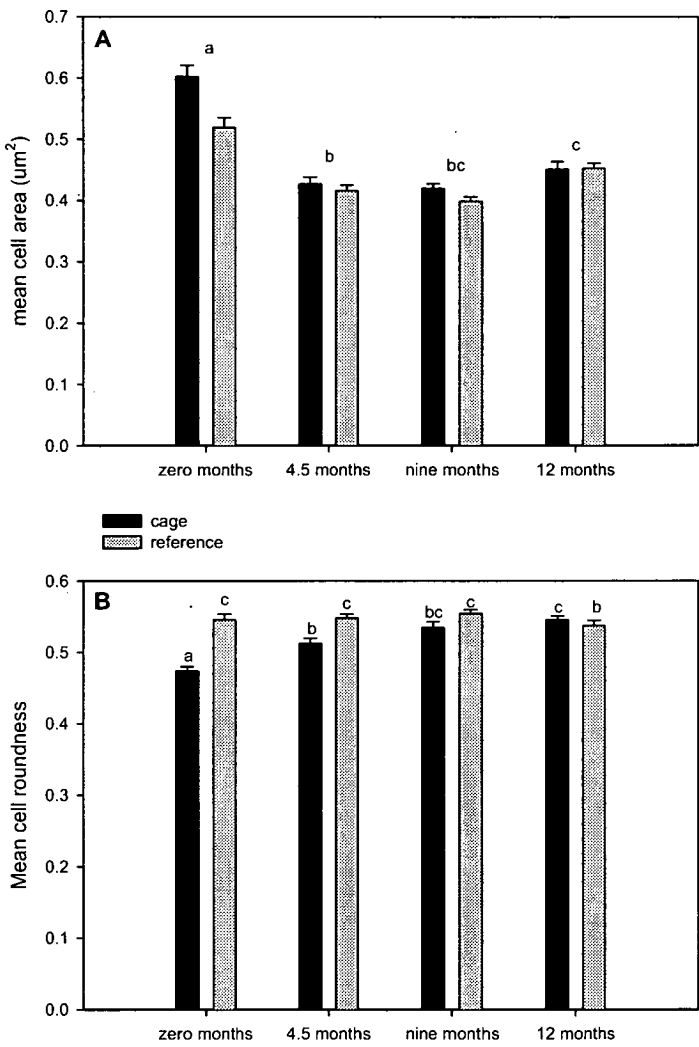


Figure 2-6 Mean size (μm^2) (A) and roundness (B) of bacterial cells in sediments from farm 1, cage 1, reference 1 over the second 12 month farm cycle. Superscripts in A refer to effects of time on cell area over the 12 month peirod, and therefore refer to the mean of cage and rfERENCE sites at each time. Superscripts in B refer to post hoc tests of treatment/time combinations. Bars sharing a common superscript are not significantly different ($p>0.05$). Bars \pm standard error.

Table 2-7 ANOVA table for mean cell roundness at farm 1, cage1, and reference 1 during the second 12 month stocking period (March 2002 – March 2003).

Source	df	Mean Square	F	Sig. (p)
Treatment	1	0.0157	33.141	0.000
Time	3	0.0044	9.269	0.000
Depth	2	1.45 X 10 ⁻⁴	0.306	0.738
Treatment x Time	3	0.0051	10.708	0.000
Treatment x Depth	2	2.67 X 10 ⁻⁴	0.564	0.573
Time x Depth	6	1.94 X 10 ⁻⁴	0.410	0.869
Treatment x time x depth	6	3.95 X 10 ⁻⁴	0.935	0.549
error	48	4.727 X 10 ⁻⁴		
Total	72			

2.3.1.6 Cellular morphology at Cage 2 and Reference 2 over farm cycle 2

A 3-way factorial ANOVA (factors as above) indicated no significant interactions or effects for treatment and depth, but a significant effect of time ($F_{4,60}=34.49, p<0.000$) on cell size (area) at cage 2 and reference 2 over the second farm cycle. Cell size significantly decreased ($p<0.05$) from $0.4691 \pm 0.012 \mu\text{m}^2$ to $0.43 \pm 0.0007 \mu\text{m}^2$ over the 12 month period.

Table 2- 8 presents ANOVA results for the effects of the above factors on cell shape at cage site 2 and reference site 2. Cell shape at the cage site became more round during the extra 1.5 months following, at which point it was the same as that exhibited by cells at the reference site. During the subsequent nine month stocking period cell shape at both reference and cage sites tended to become more elongated, before changing to become more round in the following three month following period. The shape of cells was only significantly different ($p<0.05$) between the cage and reference site at the beginning of the second 12 month cycle; cells at the cage site were more round.

Table 2-8 ANOVA table for mean cell roundness at farm 1, cage2, and reference 2 during the second 12 month stocking period (April 2002 – April 2003).

Source	df	Mean Square	F	Sig.
Treatment	1	7.36×10^{-4}	1.853	0.178
Time	4	0.012	30.611	0.000
Depth	2	4.044×10^{-5}	0.102	0.903
Treatment x Time	4	3.99×10^{-3}	10.039	0.000
Treatment x Depth	2	1.04×10^{-4}	0.262	0.770
Time x Depth	8	3.25×10^{-4}	0.818	0.590
Treatment x time x depth	8	2.61×10^{-4}	0.656	0.728
error	60	3.917×10^{-4}		
Total	90			

2.3.1.7 Bacterial Biomass

Bacterial biomass increased during the stocking period and decreased during the fallowing period (Table 2- 9). Biomass followed the same trends as total bacterial counts. Bacterial biomass did not become as high at cage sites during the second 12 month farm cycle.

Table 2-9 Bacterial biomass (µg C/g sediment) at farm 1 over both farm cycles

<i>FARM</i>	TWO	NINE	12 MONTHS
<i>CYCLE 1</i>	MONTHS	MONTHS	
Cage	40.11 ± 16.9	305.53 ± 46.9	167.86 ± 13.4
Reference	14.81 ± 1.8	36.41 ± 2.1	15.43 ± 1.2

<i>FARM</i>	ZERO	NINE	12 MONTHS
<i>CYCLE 2</i>	MONTHS	MONTHS	
Cage 1	76.82 ± 5.5	124.17±18.4	62.38 ± 11.2
Reference 1	14.37 ± 1.7	12.6 ± 1.9	7.90 ± 0.53
 Cage 2	 22.53 ± 1.0	 88.59 ± 11.0	 18.71 ± 2.5
Reference 2	25.59 ± 3.2	7.15 ± 0.4	6.60 ± 0.6

2.3.2 Farm 2

2.3.2.1 Bacterial counts

Mean microbial direct counts ranged from $2.2 \times 10^8 \pm 2.1 \times 10^7$ to $2.6 \times 10^8 \pm 1.5 \times 10^7$ cells/g sediment at reference sites and from $2.42 \times 10^8 \pm 1.7 \times 10^7$ to $5.37 \times 10^9 \pm 4.2 \times 10^8$ cells/g sediment at cage sites. Results of the three-way factorial ANOVA performed are presented in Table 2- 10.

Table 2-10 ANOVA table for log mean counts (cells/g) at farm 2 during the first 12 month stocking period (April 2001 – March 2002).

Source	df	Mean Square	F	Sig.
Treatment	1	9.343	601.058	0.000
Time	3	5.87	377.65	0.000
Depth	2	0.176	11.329	0.000
Treatment x Time	3	3.481	223.929	0.000
Treatment x Depth	2	6.41 X 10 ⁻⁵	0.004	0.996
Time x Depth	6	0.0057	0.366	0.899
Treatment x time x depth	6	0.0052	0.332	0.919
error	108	0.0156		
Total	132			

Bacterial numbers increased as farming progressed and decreased during the fallowing period (Figure 2- 6A). Bacterial numbers did not, however, return to pre-stocking or reference levels by the end of the 3 month fallowing period. Bacterial numbers did, however, return to reference levels over the following 12 months, during which time no cages were over these sites. Depth within the sediment core had a significant effect on bacterial numbers. Numbers at the surface were significantly higher ($1.9 \times 10^9 \pm 4.7 \times 10^8$ cells/g sediment) than those deepest in the core ($1.3 \times 10^9 \pm 3.2 \times 10^8$ cell/g sediment) ($p < 0.05$).

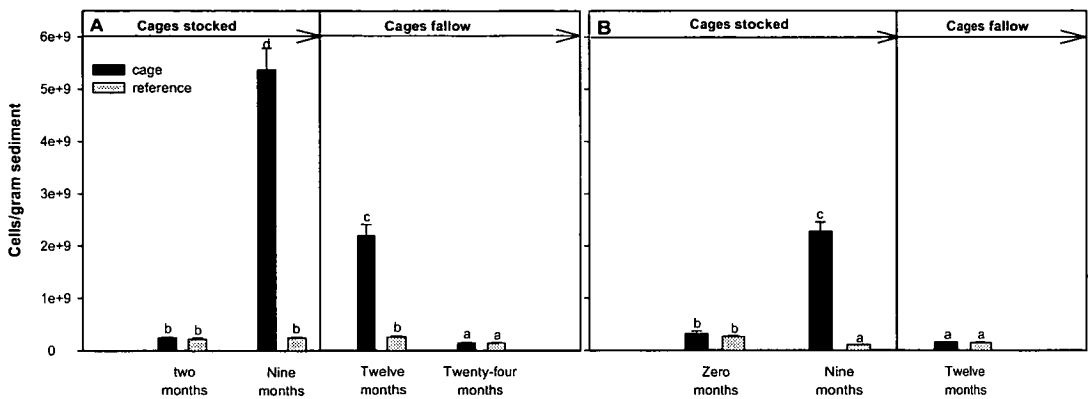


Figure 2-7 Mean bacterial numbers (cells/g sediment) at farm 2 over the two, 12-month farm cycles. A) First 12 month cycle (n=4 at two months, n=6 at remaining sampling times). B) Second 12 month cycle (n=6). Months indicate time through the respective farm cycles. Superscripts refer to Tukey’s post hoc tests of all treatment/time combinations within each graph. Bars sharing a common superscript are not significantly different (p>0.05). Bar ± standard error.

During the second 12 months the original sites were left fallow and two more stocked cages were sampled. ANOVA results from these sites are presented in Table 2- 11.

Bacterial numbers increased with farming and decreased with fallowing (Figure 2- 6B). Numbers at the cage sites were not significantly different from those at reference sites at the end of the fallow period. Again, bacterial numbers were higher at the surface ($1.6 \times 10^9 \pm 3.6 \times 10^8$ cells/g sediment, 0-2mm; $9.7 \pm 2.4 \times 10^8$ cells/g sediment, 5-10mm) of the core (p<0.05).

Table 2-11 ANOVA table for log mean counts (cells/g) at farm 2 during the second 12 month stocking period (March 2002 – March 2003).

Source	df	Mean Square	F	Sig. (p)
Treatment	1	5.074	214.682	0.000
Time	2	2.42	102.41	0.000
Depth	2	0.211	8.926	0.000
Treatment x Time	2	4.914	207.939	0.000
Treatment x Depth	2	4.93 X 10 ⁻³	0.209	0.812
Time x Depth	4	0.011	0.455	0.768
Treatment x time x depth	4	0.031	1.298	0.277
error	89	0.024		
Total	107			

2.3.2.2 Cellular morphology

A 3-way factorial ANOVA (factors as above) indicated no significant interactions or main effects except for time ($F_{2,66}=6.95, p=0.002$) on cell size at cage and reference sites over the first 12 month cycle. Cell size increased at both cage and reference sites over the 12 month period.

The results of a similar ANOVA using cell roundness as the dependent variable are shown in Table 2- 12.

Table 2-12 ANOVA table for cell roundness at farm 2 during the first 12 month stocking period (April 2001 – March 2002).

Source	df	Mean Square	F	Sig. (p)
Treatment	1	5.812 X 10 ⁻³	1.655	0.203
Time	2	0.031	8.673	0.000
Depth	2	3.01 X 10 ⁻⁵	0.009	0.991
Treatment x Time	2	0.014	4.016	0.023
Treatment x Depth	2	2.011 X 10 ⁻⁴	0.057	0.944
Time x Depth	4	6.38 X 10 ⁻⁴	0.182	0.947
Treatment x time x depth	4	6.383 X 10 ⁻⁴	0.182	0.947
error	66	0.0035		
Total	84			

An interaction between treatment and time showed that at cage sites cells became more elongated over the 9 month stocking period and remained this shape for the fallow period (Figure 2- 7). At reference sites the cells also became elongated over the 9 month stocking period, but to a greater extent than at cage sites. By the end of the fallow period cells had become more round and were not different from those at the cage site. At both the beginning and end of the 12 month period cell shape at cage and reference sites was not significantly different.

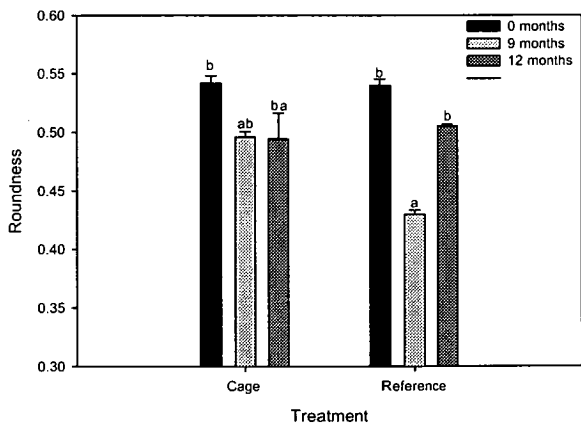


Figure2-7 Mean cell roundness at farm 2 the first` 12 month farm cycle (n=3). Superscripts refer to tukey’s post hoc comparisons of all treatment/time combinations. Bars sharing a common superscript are not significantly different (p>0.05). Bar ± standard error.

Over the second 12 month farm cycle a 3-way factorial ANOVA again indicated no significant interactions or main effects, except for time ($F_{2,71}=5.75, p=0.005$), on cell size at cage and reference sites. Cell size decreased significantly over the 9 month stocking period (from $0.6\text{ }\mu\text{m}^2$ to $0.45\text{ }\mu\text{m}^2$) and remained at this smaller size over the following 3 month fallowing period.

As with cell size, cell shape was only affected by time ($F_{2,71}=11.28, p<0.000$). Cell shape became more round at both cage and reference sites over the second 12 month cycle.

2.3.2.3 Bacterial biomass

As at farm 1, bacterial biomass increased significantly during the stocking period and decreased during the fallow period (Table 2- 13). Biomass at cage sites was not as high during the second 12 month period.

Table 2-13 Bacterial biomass ($\mu\text{g C/g sediment}$) over at farm 2 over both farm cycles

FARM	TWO	NINE	12 MONTHS	24 MONTHS
CYCLE 1	MONTHS	MONTHS		
Cage	20.82 ± 1.9	476.37 ± 36.4	234.78 ± 25.77	11.22 ± 0.85
Reference	11.26 ± 1.15	24.38 ± 1.9	27.76 ± 2.7	10.60 ± 0.77
FARM	ZERO	NINE	12 MONTHS	
CYCLE 2	MONTHS	MONTHS		
Cage	28.09 ± 3.2	166.53 ± 21.7	13.57 ± 0.92	
Reference	27.76 ± 2.7	9.45 ± 0.78	10.60 ± 0.77	

2.4 Discussion

Microbial numbers encountered in this study are similar to those reported previously for marine sediments and organically impacted marine sediments (Albertelli, Covazzi, Danovaro, Fabiano, Frascchetti, Pusceddu, 1999; Findlay, Watling, Mayer, 1995; Rysgaard, Thamdrup, Risgaardpetersen, Fossing, Berg, Bondo Christensen, Dalsgaard, 1998; Vezzulli, Chelossi, Riccardi, Fabiano, 2002). For sediments receiving a high organic load an increase in microbial numbers and activity has

generally been reported, although La Rosa *et al.* (2004) did not observe an increase in microbial numbers due to fish-farming. This may be due to the presence of scavenging fish under their farm. These scavenging fish clear the sedimenting organic matter before it becomes available to benthic communities. Felsing *et al.* (2002) also found that the impacts of sea-cage farming were ameliorated by scavenging fish. The salmon farms under investigation here do not possess communities of scavenging fish and so have been observed to exhibit the more generally reported response of increased microbial numbers with organic loading.

2.4.1 Microbial numbers

Bacterial numbers were higher at the surface (0-2mm) than they were in the deepest layer (5-10mm), regardless of treatment, although there was often no statistically detectable difference between the middle layer and the other two. I suspect the latter is an artefact of the imprecise means of sectioning the cores, the surfaces of which were often uneven. The middle (2-5mm) layer acted more as a buffer to separate the other two layers than as a layer in its own right. This result suggests that bacterial growth rates are determined primarily by the availability of nutrients (Bastviken, Ejlertsson, Tranvik, 2001; Blume, Bischoff, Reichert, Moorman, Konopka, Turco, 2002) and not the pathways available for growth. It has been previously reported that waste from fish-farms contains highly labile organic matter (McGhie, Crawford, Mitchell, O'Brien, 2000). This organic matter is first available to microbes at the sediment surface, which utilise easily metabolised substrates, while less labile matter passes to the underlying sediments. If metabolic pathways, such as oxic respiration, were of primary importance to bacterial production, then as the sediment layers become more homogeneously anoxic (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004) so too should numbers become more evenly distributed throughout the core. Oxic-zone depths were shown to be significantly shallower at the cage sites than at the reference sites (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004), but bacterial numbers were higher at the cage sites, further highlighting the importance of nutrient supply, rather than oxygen availability, in determining bacterial densities.

The effect of bioturbation must also be considered. The cage sediment faunal communities investigated in this study became dominated by *Capitella* spp. (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). These organisms are often associated with organically enriched sediments and via bioturbation are thought to exert some control over the distribution of bacteria in sediments (Wu, Tsutsumi, Kita-tsukamoto, Kogure, Ohwada, Wada, 2003). The burrowing activity of *Capitella* sp. (and other benthic fauna) can stimulate benthic production and lead to localized proliferation of bacteria around sediment structures such as burrows (Wu, Tsutsumi, Kita-tsukamoto, Kogure, Ohwada, Wada, 2003). Although bioturbation may lead to the creation of localized oxic zones, oxygen penetration in the sediment mass, as a whole, declines with organic loading. This observation is certainly true for the sediments in this study (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). Bioturbation increases the heterogeneity of the sediment microbial community, making accurate assessments of mean microbial population parameters problematic. This difficulty can, however, be overcome by ensuring adequate replication and increased sampling effort. In this study, employing an image analysis system ensured many more cells were counted than would have been possible without such a system. Although greater resolution would have been obtained by increasing replication further, the sampling design used here was adequate. In these sediments, then, it appears that the supply of labile organic matter is the primary determinant of microbial biomass and production. This is observed as greater microbial numbers at the surface, the deposition point for fresh, labile organic matter.

Generally, at farm 1 bacterial numbers and biomass increased with organic loading and then declined during fallowing. This trend is clearest in the first 12 month cycle, but persists throughout the trial. It should be noted that during this first cycle the large amount of the variation seen at the end of the stocking period may be attributed to the different levels of loading experienced by each replicate cage during this time (see chapter 1.4.2). Bacterial numbers under Cage 1 reach higher levels than those at Cage 2, and farm records indicate that Cage 1 was stocked and fed more consistently over the nine month period than Cage 2 (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). Despite this the impact of organic loading on microbial numbers was still detected. It has been reported previously (La

Rosa, Mirto, Mazzola, Danovaro, 2001) that microbiological parameters are very sensitive to fish-farm biodeposition. Indeed it has been reported that the microbial response to organic loading is rapid in natural (undisturbed) microbial communities as well (Rysgaard, Thamdrup, Risgaardpetersen, Fossing, Berg, Bondo Christensen, Dalsgaard, 1998). Although microbial numbers in this study did not decline with fallowing to the same extent as those reported by La Rosa *et al.* (2001), initially there was a rapid decline in numbers as the highly labile organic matter was utilised (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). This rapid decline would occur any time loading was decreased (during harvesting events, if feeding was stopped etc.), not only if loading was halted for extended periods, as in fallowing. The rapidity of the microbial response highlights the importance of sampling quickly after the cessation of loading if a fair assessment of impact is to be made.

A similar pattern is evident during the second cycle and because the cages were treated as separate treatments (due to the different timing of fallowing periods) an interesting result emerged. The extra 1.5 months fallowing afforded Cage 2 resulted in a significant decline in bacterial numbers over that period. This suggests that with the level of loading experienced at this site, three months was not long enough for the highly labile organic matter deposited to be utilised. Cage 2 also appeared to recover more fully during the second fallow period than Cage 1 (Figures 2A & 2B). The extra fallowing time given to Cage 2 may have been long enough for the utilization of the more labile organic matter, allowing the sediment microbial community to “catch-up” more fully before the second farm cycle.

This result indicates that there is not necessarily a cumulative effect of farming on bacterial numbers. Numbers were elevated after the first cycle, but decreased by the end of the second cycle. It is important to note the high variability and the fact that the initial samples were taken after two months farming. The first sample is then, probably elevated above the true pre-stocking level. It should also be noted that both farms were run less intensively (lower biomass and lower feed input) during the second 12 month cycle (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). The fact that bacterial numbers and biomass were not as high during the second cycle highlights the importance of farm management practices

in mitigating/preventing the impacts of farm loading. Lower inputs allow the benthic community to keep pace with organic loading.

At farm 2, the response of bacterial numbers to farm loading was similar to that at farm 1. Numbers increased throughout the nine month stocking period and declined over the three month fallow period during both farm cycles. Over the first farm cycle bacterial numbers did not return to reference site levels, but did continue to decline over the following 12 months of no loading. As was the case at farm 1, the farming intensity was higher during the first 12 month cycle. Over the second cycle, bacterial numbers at cage sites were not different from those at reference sites at the beginning or end of the cycle, but were elevated at the end of the stocking period. This result further supports the conclusion that microbial numbers respond rapidly and directly to labile organic input. During farm cycle 2, farming intensity was less than during the first cycle (chapter 1.4.2). As a result bacterial numbers were not as high at the end of the second stocking period. Less labile carbon was available to support rapid microbial growth in the second 12 month cycle and consequently microbial numbers did not increase to the same extent that they did in the first cycle, used the more limited (but still substantial) carbon and declined to levels approaching those at reference sites.

The count also shows small, but statistically significant, changes in bacterial numbers in the reference site sediments over the trial at both farms (Figures 2- 2 and 2- 6). These changes indicate the natural heterogeneity and dynamic nature of the sediment bacterial communities. Bacterial numbers respond to events such as seasonal temperature changes and storm and sedimentation events. The fact that the changes in bacterial numbers at reference sites are small in comparison to those observed at the cage sites further supports the idea that, although many factors influence bacterial numbers, in these sediments at least the major limiting factor is nutrient availability.

2.4.2 Cell size morphology and microbial biomass

Results regarding cell size and morphology are not as easily interpretable. It has been reported previously (Cotner, Ogdahl, Biddanda, 2001) that more active cells are larger than less active cells. Given an increase in nutrients and an increase in cell number it

was expected that a concomitant increase in cell size might occur. It was also thought that cell shape might be an indicator of population stability under organic loading. Cell morphology is a phenotypic expression of cellular genetic information, therefore a shift in community cell shape may be indicative of a change in bacteriotypes (Liu, Dazzo, Glagoleva, Yu, Jain, 2001). No such clear trends were observed. The reasons for this are that both cell size and morphology are dependant on several factors, not merely activity and cell type.

The utility of cell size and morphology estimates to ascertain community shifts has yet to be proven in natural bacterial assemblages. Using morphological diversity as an indicator of microbial community structural change is more plausible if cells are actively growing and not in a state of dormancy (Liu, Dazzo, Glagoleva, Yu, Jain, 2001). A change in cell size would only be evident as an indicator of more active cells if the bacterial type didn't shift. It has also been observed that in natural assemblages a very high proportion of bacterial cells are dead or dormant (Amann, Ludwig, Schleifer, 1995; Gasol, del Giorgio, Massana, Duarte, 1995; Luna, Manini, Danovaro, 2002). These dormant cells are often very small and gaining reliable morphology data can be difficult. Cole *et al.* (1993) reported that cell size may be influenced heavily by bacterivory. Cells are larger when bacteriovores are absent. For these reasons, attributing changes in cellular characteristics to any one cause is often not possible in natural assemblages. Furthermore, the analysis system employed in this study only assessed cell morphology as roundness. In extremely complex systems such a simple system may not be elaborate enough to assess complex changes. The Centre for Microbial Ecology Image Analysis System (CMEIAS) (Liu, Dazzo, Glagoleva, Yu, Jain, 2001) has been developed to assess the morphological diversity of 40 morphotypes in complex communities. A system such as this may be more applicable to detecting community shifts in complex systems than the simple one employed here, but it is not yet available for general use and it too has only been tested on cultures and fast growing, nutrient-amended bioreactor systems.

The accurate assessment of bacterial cellular parameters is not only important for the purposes of assessing community diversity. Accurate assessment of these parameters is necessary for the determination of microbial biomass in terms of microbial carbon.

Bacteria convert organic carbon to biomass, provide a grazing crop to higher trophic levels, and thus structure the microbial loop in benthic ecosystems (La Rosa, Mirto, Mazzola, Danovaro, 2001). The method used in this study assessed the characteristics of over 36×10^4 cells at two farms to determine the effect of organic loading on bacterial biomass. Far more cells than could have been assessed using non-automated methods. Biomass was shown to increase rapidly under cages while farming progressed and to decrease rapidly after cessation of farming. Although the sampling times utilised in this study did not allow rigorous assessment of seasonal variability in biomass data, biomass did respond more strongly to farm input than to variations in seasonal abiotic parameters. Bacterial biomass has been linked to nutrient fluxes previously (Deming, Baross, 1993) and the above results further suggest that in these sites bacterial biomass and production are limited primarily by nutrient availability, rather than seasonal factors such as temperature or by metabolic pathways available for growth. High bacterial numbers also suggest that increased substrate availability has prevailed over the potential limiting effects of an extreme environment of reduced O_2 , low redox potential and high sulphide concentrations.

2.5 Conclusions

Microbial numbers and biomass increased with fish-farm load and decreased with fallowing. The length of the fallowing period and the amount of farm input (observed as the intensity of farming) appeared to be important factors in the speed with which sediments recovered. These findings were consistent with those previously reported (for example (Albertelli, Covazzi, Danovaro, Fabiano, Frascetti, Pusceddu, 1999; Findlay, Watling, Mayer, 1995; Grenz, Hermin, Baudinet, Daumas, 1990; La Rosa, Mirto, Mazzola, Danovaro, 2001; La Rosa, Mirto, Mazzola, Maugeri, 2004; Vezzulli, Chelossi, Riccardi, Fabiano, 2002)), who also showed an increase in microbial numbers with fish-farm impact. Other benthic indicators investigated in these sediments also appear to follow similar patterns of impact and recovery (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). Farm impact on microbial numbers did not appear to be cumulative to any great extent at farm 1, where numbers were monitored at the same sites over two production cycles.

The impact of the farm sites was localised. As has been reported previously (Beveridge, 1996; Karakassis, 1998; Macleod, Crawford, Moltschaniwskyj, 2004) the impact of the farms, in terms of changes to microbial biomass, were not evident at reference sites, which were located 150m away.

Some microbiological parameters (biomass and total count estimates) appear to be highly sensitive to organic loading. The morphological parameters measured in this study are not as easily interpretable in terms of their response to farm loads. This should not preclude them from future studies, but does indicate the need to refine the methods utilised to make them more sensitive to subtle variations.

Chapter Three

Microbial Diversity in sediments at two salmon farms

Chapter 3 Microbial Diversity at two Salmon Farms

3.1 Introduction

Clone library construction has been used to investigate the microbial diversity of various sediments in several previous studies (for example, (Bowman, McCammon, Brown, Nichols, McMeekin, 1997; Bowman, McCuaig, 2003; Bowman, McCammon, Gibson, Robertson, Nichols, 2003; Gray, Herwig, 1996; Ravenschlag, Sahm, Pernthaler, Amann, 1999)). These studies have provided much information, that is unavailable to culture-based methods, on the phylotypes that inhabit sediments and the massive diversity that sediments harbour. One of the findings that is common to all sediment 16S rDNA clone library studies is that sediments are extremely diverse and the amount of sampling effort necessary to increase coverage to a level ensuring most phylotypes are sampled is not practically achievable, especially with any statistically valid level of replication (Kemp, Aller, 2004). Despite this, clone libraries provide a means to sample a large number of microbial phylotypes that would otherwise be unknowable and provide a starting point for developing more targeted analyses. These previous studies have demonstrated that the most abundant phylotypes present in marine sediments are members of the gamma and delta-proteobacteria, the CFB group and Planctomycetales, but also that many other bacterial phylotypes are present. Most of this research has been concentrated on more extreme environments (e.g. cold sediments, deep-sea sediments and hydrothermal vents). However, there is a growing awareness of the importance of near-shore coastal areas as receiving grounds for anthropogenic input.

The commercial production of Atlantic salmon has become an increasingly important industry in Tasmania and has continued to increase production and expand. With this comes an increasing awareness of the importance of protecting in-shore coastal areas from anthropogenic impact. Intensive cage culture of salmonid species leads to localized organic input to the underlying sediments. The impact of this organic loading on the microbial community is largely unknown. It is known, however, that organic loading causes both chemical and physical changes to sediments and sediment infaunal communities. Bacterial communities are inextricably linked to their environment, so these changes may be expected to cause changes to both microbial

community structure and function. Without an understanding of microbial community reaction to organic perturbation it is difficult to predict the effect of organic input on nutrient cycling, sediment fauna and microbial diversity.

Clone libraries have been constructed from four sites (two salmon farms of differing sediment type and adjacent control sites) to gain an insight into the natural diversity in sediments unaffected by organic carbon inputs and under fish-farms. The libraries are also compared to elucidate the role of organic pollution in contributing to microbial community shifts.

3.2 Methods

3.2.1 Sample collection

Sediments were collected as described in chapter 1.4.4.

3.2.2 DNA extraction

Bacterial genomic DNA was obtained by the bead beating method, modified from Purdy (1996). Samples of sediment (0.5 g) were aliquoted into 2-ml screw-cap Eppendorf tubes, each with 0.5 g of 0.1-mm-diameter glass beads baked at 260°C. The following solutions were then added to each tube: 0.70 µl of 120 mM sodium phosphate (pH 8.0) plus 1% (wt/vol) acid-washed polyvinylpyrrolidone, 500 µl of Tris-equilibrated phenol (pH 8.0), and 50 µl of 20% (wt/vol) sodium dodecylsulfate. The samples were bead beaten (Mikrodismembrator U; B. Braun Biotech International, Melsungen, Germany) three times at 3800 rpm for 10 s, with 30 s on ice in between bead beatings. They were then centrifuged at 20,800g for 2 min, and the supernatants stored on ice. In order to extract residual nucleic acid from the sediment pellet, the pellet was resuspended in 700 µl of 120 mM sodium phosphate, pH 8.0, and bead beaten at 3800 rpm for 20 s, and then centrifuged again. The supernatants from both the first and second extractions were pooled. Nucleic acid was precipitated with 2 volumes of ethanol and 1/10 volume of 3M sodium acetate. After centrifugation at 20,800g for 30 min, the supernatant was removed by aspiration and the pellet was washed with 70% ethanol and resuspended in 50 µl of MilliQ water.

Extractions were then purified using the Prep-A-Gene DNA (Bio-Rad Laboratories, CA, USA) purification system, as per the manufacturer's instructions. DNA extracts were visualised on 1% agarose gels containing 10 µg/ml ethidium bromide using UV transillumination. Final extractions (50 µl) were then stored at -20°C.

3.2.5 Clone Libraries

3.2.5.1 Clone library construction and comparison

Clone libraries were constructed for six sediment samples; Farm 1, Cage 1 sediment at the end of the first stocking period (nine months, year 1) and at the end of the first fallow period (12 months, year 1), Farm 2, cage 1 at the end of the first stocking period (nine months, year 1) and at the end of the first fallow period (12 months, year 1) and at reference site 1 of both farms at nine months of the first year of the trial. These libraries will be referred to as F1C9, F1C12, F2C9, F2C12, F1R9 and F2R9 respectively. The universal 16S rDNA primers 519f (5'-CAGCMGCCGCGGTAATAC-3') (Lane, 1991) and 1492r (5'-TACGGYTACCTTGTTACGAC-3') (Lane, 1991) were used to amplify 16S rDNA fragments from prokaryotes from the sediment DNA. The amplified fragment was then purified using the Prep-A-Gene kit (Bio-Rad). The fragment to be cloned was ligated into the pGEM-T vector (Promega) according to the manufacturer's instructions and transformed into Epicurian coli XL ultracompetent cells (Stratagene). Transformants were screened using blue-white selection on Luria agar containing Xgal/IPTG and 100 µg/ml ampicillin. White colonies were then transferred to fresh plates and reincubated overnight. Plasmids were extracted using the Ultraclean miniplasmid extraction kit (MoBio). Positive clones were sequenced in one direction with the Beckman ready reaction dideoxy cycle sequencing kit (Beckman) and M13 forward primers, and sequencing was performed with the Beckman CEQ2000XL automated capillary sequencing system.

The chimera-check tool of the Ribosomal RNA Database Project

(<http://rdp.cme.msu.edu> (Maidak, Cole, Lilburn, Parker, Saxman, Farris, Garrity, Olsen, Schmidt, Tiedje, 2001)) was used to check possible chimeric sequences.

Sequences were aligned with reference sequences obtained from the National Centre for Biotechnology Information (NCBI) nucleotide database (Altschul, Madden, Schäffer, Zhang, Zhang, Miller, Lipman, 1997) using BioEdit (version 5.0.9) (Hall, 1999). Similarity trees were created by calculation of maximum-likelihood distances and by using the neighbour-joining algorithm through the BioEdit program). Trees were created from the NEIGHBOR output by using the program TREEVIEW. 16S rDNA sequences from *Thermotoga maritimum* and *Coprothermobacter platensis* were used as outgroup references on all trees.

Clones with a sequence similarity of >98% were considered to be the same phylotype (Keswani, Whitman, 2001) for the purposes of calculating diversity statistics. Trees generated, however, are not limited to this cut-off. Simpson's index, the Shannon-Wiener index and Margalef's index were calculated and the Chao-1 estimator (<http://www2.biology.ualberta.ca/jbrzusto/rarefact.php>) was used to calculate species richness. The method of Singleton *et al.* (2001) was used to compare the similarity of clone libraries directly. This method utilises the LIBSHUFF computer programme (<http://www.arches.uga.edu/~whitman/libshuff.html>) to generate homologous and heterologous coverage curves from clone libraries, which are then compared. The DNADIST programme of PHYLIP (<http://evolution.genetics.washington.edu/phylip.html>) using the Jukes-Cantor model for nucleotide substitution was used to construct the distance matrix submitted to LIBSHUFF.

3.3 Results

3.3.1 Construction of bacterial clone libraries

Clone libraries were successfully generated for each sediment. Table 3- 2 contains clone number and diversity index information for each library. Sequences obtained from clone libraries were divided into 6 groups for ease of handling. Figure 3- 1 shows the percentage contribution of each group to clone library composition.

At farm 1 cage sites the CFB group comprise a higher proportion of clones at the end of the stocking period than at the end of the fallowing period. The opposite is true of the delta and alpha-proteobacterial groups, which appear to increase during the fallowing period.

At farm 2, the major difference in clone group proportions is the disappearance of the alpha group bacteria and the appearance of a few archaeal sequences during the fallowing period. The other groups remain relatively constant throughout the three month sampling period.

Reference site libraries are dominated by gamma and delta-proteobacterial sequences, and by non-proteobacterial sequences. Very few sequences from the epsilon and alpha groups were observed, but the CFB group were well represented. Chapter 3.3.2 presents more detailed descriptions of sequence types present in libraries.

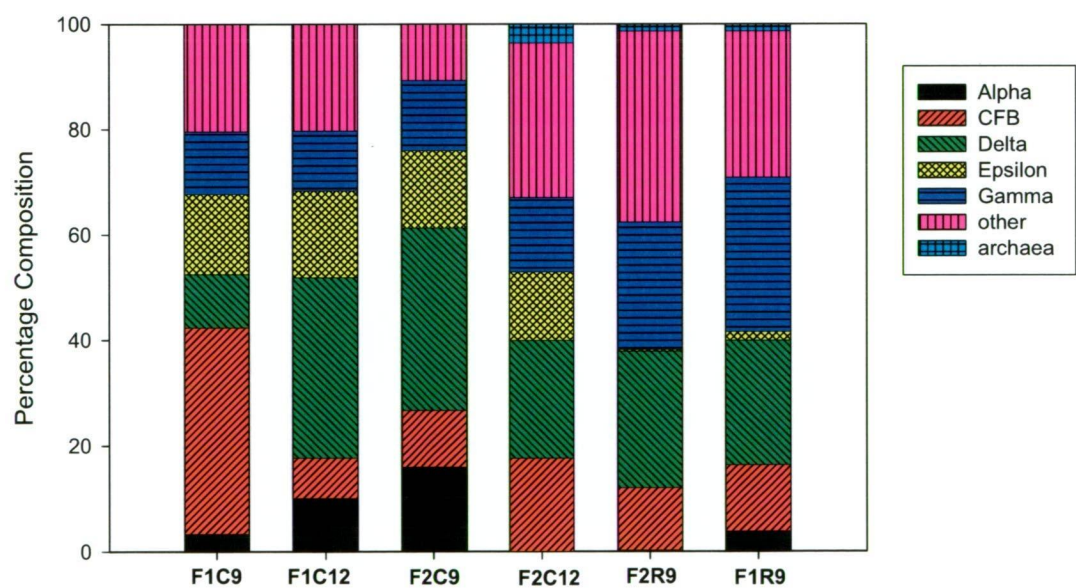


Figure 3-1 Clone library composition. Bars represent the percentage of each group to clone library composition.

3.3.2 Phylogeny of clone libraries

3.3.2.1 Alpha and epsilon-proteobacteria

Clones from all libraries were spread throughout both the alpha and epsilon-proteobacteria, with no clear association based on farm or sampling time (Figure 2). Within the alpha-proteobacteria the distribution of clones is not even within the site libraries. No clones from the F1C9 library were from the alpha-proteobacteria. The clones fell into two major clusters. Sequences in the *Sphingopyxis* cluster were only seen at farm one, and primarily at the reference site, with one other sequence from the F1C12 library. The *Rhodobacter/Roseobacter* cluster comprised sequences from all libraries except F1C9. The alpha-proteobacteria displayed considerable diversity, even though most clones were concentrated in the *Rhodobacter/Roseobacter* clade.

Clones that grouped with the epsilon-proteobacteria grouped into a single cluster, and were well represented in cage site libraries (Figure 3- 1). The cluster included environmental clones derived from a variety of anoxic and deep-sea sediments and hydrothermal vents. Only one reference site clone (F1R9 67) was found to group outside this cluster.

3.3.2.2 Delta-proteobacteria

Delta-proteobacterial phylotypes showed wide diversity fell largely into seven phylotypes (Figure 3a-3b). The two largest groups were the *Desulfobulbaceae* and *Desulfosarcina* groups (Figure 3b). Both groups included clones from a range of sediments including cold, coastal and methane seep sediments and also included several species known to be sulphate reducers. Several clones grouped with the *Desulfuromonas* group, which includes sulphur and iron-reducing bacteria. Clones associated with Myxobacteria, some of which have been shown to be capable of anaerobic growth (Sanford, Cole, Tiedje, 2002), were well represented among the reference site libraries, but only a single clone from the cage libraries (F2C9 A05) was observed (Figure 3a). The reference site libraries were well represented in all groups, but some separation of clones from the cage sites was observed. Two groups consisted of only clones; JTB38/CLEAR9 and Eel-TE1A4. These groups comprised

clones from the reference site libraries and from the F2C12 library. No clone from F1C libraries were observed to associate with these groups.

3.3.2.3 Gamma-proteobacteria

The gamma-proteobacteria were very widely sampled and showed great diversity (Figures 4a and 4b). A number of clones were associated with chemoheterotrophic bacteria that inhabit a broad range of sediment systems including *Colwellia*, *Halomonas*, *Moritella*, *Oceanospirillum*, *Photobacterium*, *Pseudoalteromonas*, *Pseudomonas*, *Psychromonas* and *Shewanella*. Although *Beggiatoa* sp.-like filamentous bacteria were observed in the cage site sediments, only three clones were associated with this group, one from the F1C9 library and two from the F1R9 library (Figure 4a). Several clones were distantly associated with the *Ectothiorhodospiraceae* group (Figure 4b). The majority of gamma-proteobacterial phylotypes, however, formed five clusters that were distinct from cultured species (Figures 4a and 4b) and included clones previously sampled from marine sediments. The most significant group (BD3-6/JTB255 group) in the reference samples included clones from a broad range of sediments from Antarctic lake sediments to coastal sea grass sediments. Only one cage clone (F1C12) was found in this group. The B2M60 and ASW98-7e groups also included clones from diverse sediments and included clones from both reference and cage sediments. The BPC036 group, which is associated with methane seeps, contained only clones from the reference sites of this study. The JTB148/Sva0091 group formed a distinct group among free living and endosymbiotic sulphur oxidisers (Figure 4b) and only included reference clones.

3.3.2.5 CFB and Chlorobia

Sequences that grouped within the CFB and Chlorobia (green sulphur bacteria) showed large diversity and clustered predominantly with other uncultured environmental sequences. Five sequences grouped within the Chlorobia (three from the reference libraries and two from cage site libraries), but were not very closely related to the phototrophic *Chlorobium* cluster (Figure 5). They may represent nonphototrophic members of this group.

The sequences from the reference site libraries (F1R9 and F2R9) are distributed throughout the whole tree and do not show any zonation based on sediment type/site (Figure 5). However, the cage site libraries fall predominantly into four clusters: the *Cytophaga fermentans* group, the *Cellulophaga* group, the JTB248/Nb-1 group and the *Polaribacter/Tenacibaculum* group (Figure 5). The F2C9 sequences all grouped with the predominantly anaerobic and facultatively aerobic heterotrophic bacteria of the *C. fermentans* group, except for one clone which was associated with the genus *Polaribacter*. Sequences from the F2C12 library were also found within this group, as well as within the JTB248/Nb-1 group, and the genus *Polaribacter*.

Sequences from the F1C9 and F1C12 libraries were distributed throughout the tree, but only sequences from the F1C9 library were found in the *Cellulophaga* and *Zobellia* groups (Figure 5).

3.3.2.6 *Acidobacteria* and relatives

Clone abundance of *Acidobacteria* and other phylogenetic clusters not associated with cultured bacterial groups were evenly distributed throughout the reference sites libraries, but showed some zonation between the farm libraries (Figure 6). Clones fell into three major clusters (Figure 6). One small cluster comprised F2R9 and a single F2C9 clone, which grouped with several environmental clones from diverse sediments including OPB2, from a hot spring, and Clear-32, from Antarctic sediment. Only clones from the F2R9 library fell within the *Nitrospira* group and these comprised only two clones. The largest group comprised clones from both reference libraries, but only from F2 cage libraries (F2C9 and F2C12) and clustered with the deep branching *Nitrospina gracilis*, often placed in the delta-proteobacteria. *N. gracilis* is an aerobic, nitrifier and it is possible that the phylotypes clustering in this group have a similar capability. No F1C9 or F1C12 clones were observed in this group. Clones from this group were associated with other environmental clones from various sediments including Antarctic, anoxic, deep sea, marine, brackish and freshwater sediments, as well as soil samples.

3.3.2.7 *Planctomycetales* and relatives

The *Planctomycetales* and *Verrucomicrobia* observed in this study were very diverse. Reference library clone types were distributed evenly throughout these phyla. Cage site libraries were less well represented, but appeared to be fairly evenly distributed also (Figure 7). Clones fell into five broad groups (Figure 7). The first group comprised several clones that were associated with the anaerobic ammonia-oxidising (ANAMMOX) group. This group comprised clones from both reference libraries and the F2C12 library, but neither cage library at the end of the stocking period. The next group contained reference site clones and a single cage library clone. The BD2-16 group has previously been associated with environmental clones from deep within sediment cores (Bowman, McCuaig, 2003). The largest group was associated with the marine heterotrophic genus *Pirellula* and the species *Planctomyces brasiliensis*. This group contained clones from both cage and reference libraries, but not from cage libraries at nine months.

The BD2-18 group contained clones from both reference libraries and from both cage libraries at nine months. Finally, the *Verrucomicrobium* group comprised clones from all libraries, which were most closely associated with Antarctic sediment clones.

3.3.2.8 Other bacterial groups

The remaining phylogenetic groups comprised a diverse range of clones, among which the reference libraries were well distributed (Figure 8). The cage libraries were reasonable well represented in these groups. Phylotypes generally fell into four major clusters (Figure 8). The *Actinobacteria* contained clones previously detected in marine sediments, including those from the JTB31 group. Members of the low-G+C g positive bacteria were less abundant, but still well represented in the reference libraries. The final cluster was associated with the *Chloroflexi* group. Clones from this study did not group with cultured phylotypes from this group, but were strongly associated with environmental clones from methane seeps, Antarctic sediments and hydrothermal vents.

As indicated in figure 3-1 a small number of archaeal clones were found in several libraries (13 clones total). Few clones clustered with cultured species, but did affiliate with previously found environmental clones from marine sediments. Several clones clustered with clone PENDANT-33 (Bowman, Rea, McCammon, McMeekin, 2000), which was found to have two unique inserts between bases 793 and 794 and 1092 and 1093 (Bowman, Rea, McCammon, McMeekin, 2000). The clones from study that affiliated with this group (F1R9 71A, F1R9385, F1R9 78 and F2C12 F08) similarly possessed this insert, suggesting further that the inserts are unlikely to be PCR-artifacts. Four clones (F2C12 E08, F2R9 222, F2R9 311 and F2R9 322) affiliated with the *Crenarchaeaota*.

Legends for Figures 2 – 8

Figure 2. 16S rDNA similarity tree showing positions of members of the alpha and epsilon proteobacteria found within the marine sediments in this study. Red type denotes farm 2 (F2C9 = farm 2, cage site at 9 months, F2C12 = farm 2, cage site at 12 months), blue type denotes Farm 1 (F1C9 = farm 1, cage site at 9 months, F1C12 = farm 1, cage site at 12 months) and green type denote reference site clone (F1R9 = farm 1 reference site at 9 months, F2R9 = farm 2 reference site at 9 months). ** indicates the 9 month sampling period.

Figure 3a. 16S rDNA similarity tree showing positions of members of the delta proteobacteria found within the marine sediments in this study. Red type denotes farm 2 (F2C9 = farm 2, cage site at 9 months, F2C12 = farm 2, cage site at 12 months), blue type denotes Farm 1 (F1C9 = farm 1, cage site at 9 months, F1C12 = farm 1, cage site at 12 months) and green type denote reference site clone (F1R9 = farm 1 reference site at 9 months, F2R9 = farm 2 reference site at 9 months). ** indicates the 9 month sampling period.

Figure 3b. 16S rDNA similarity tree showing positions of members of the delta proteobacteria found within the marine sediments in this study. Red type denotes farm 2 (F2C9 = farm 2, cage site at 9 months, F2C12 = farm 2, cage site at 12 months), blue type denotes Farm 1 (F1C9 = farm 1, cage site at 9 months, F1C12 = farm 1, cage site at 12 months) and green type denote reference site clone (F1R9 = farm 1 reference site at 9 months, F2R9 = farm 2 reference site at 9 months). ** indicates the 9 month sampling period.

Figure 4a. 16S rDNA similarity tree showing positions of members of the gamma proteobacteria found within the marine sediments in this study. Red type denotes farm 2 (F2C9 = farm 2, cage site at 9 months, F2C12 = farm 2, cage site at 12

months), blue type denotes Farm 1 (F1C9 = farm 1, cage site at 9 months, F1C12 = farm 1, cage site at 12 months) and green type denote reference site clone (F1R9 = farm 1 reference site at 9 months, F2R9 = farm 2 reference site at 9 months). ** indicates the 9 month sampling period.

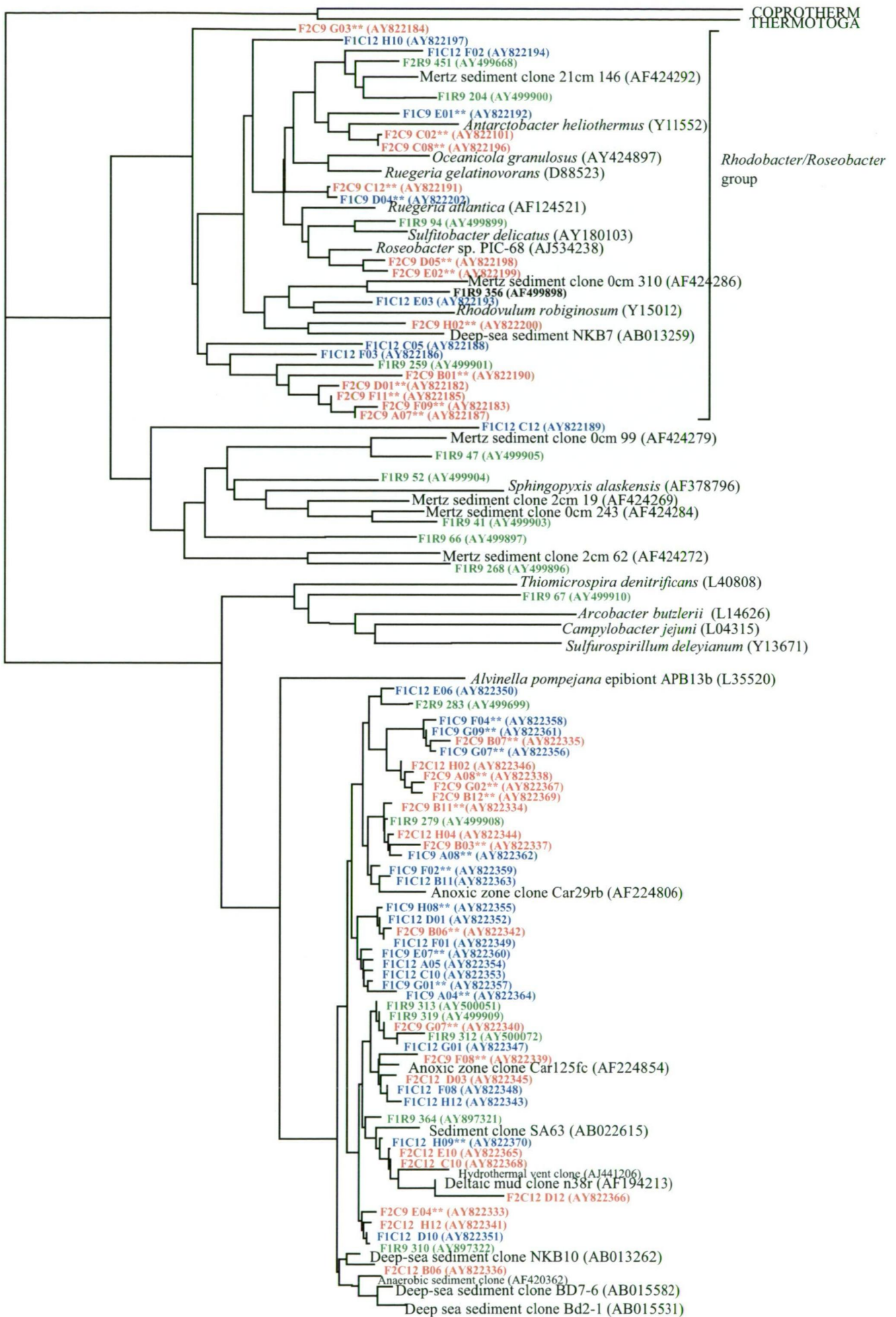
Figure 4b. 16S rDNA similarity tree showing positions of members of the gamma proteobacteria found within the marine sediments in this study. Red type denotes farm 2 (F2C9 = farm 2, cage site at 9 months, F2C12 = farm 2, cage site at 12 months), blue type denotes Farm 1 (F1C9 = farm 1, cage site at 9 months, F1C12 = farm 1, cage site at 12 months) and green type denote reference site clone (F1R9 = farm 1 reference site at 9 months, F2R9 = farm 2 reference site at 9 months). ** indicates the 9 month sampling period.

Figure 5. 16S rDNA similarity tree showing positions of members of the Flavobacteria and Chlorobia found within the marine sediments in this study. Red type denotes farm 2 (F2C9 = farm 2, cage site at 9 months, F2C12 = farm 2, cage site at 12 months), blue type denotes Farm 1 (F1C9 = farm 1, cage site at 9 months, F1C12 = farm 1, cage site at 12 months) and green type denote reference site clone (F1R9 = farm 1 reference site at 9 months, F2R9 = farm 2 reference site at 9 months). ** indicates the 9 month sampling period.

Figure 6. 16S rDNA similarity tree showing positions of members of the Acidobacteria and relatives found within the marine sediments in this study. Red type denotes farm 2 (F2C9 = farm 2, cage site at 9 months, F2C12 = farm 2, cage site at 12 months), blue type denotes Farm 1 (F1C9 = farm 1, cage site at 9 months, F1C12 = farm 1, cage site at 12 months) and green type denote reference site clone (F1R9 = farm 1 reference site at 9 months, F2R9 = farm 2 reference site at 9 months). ** indicates the 9 month sampling period.

Figure 7. 16S rDNA similarity tree showing positions of members of the Planctomycetes and Verucomicrobia found within the marine sediments in this study. Red type denotes farm 2 (F2C9 = farm 2, cage site at 9 months, F2C12 = farm 2, cage site at 12 months), blue type denotes Farm 1 (F1C9 = farm 1, cage site at 9 months, F1C12 = farm 1, cage site at 12 months) and green type denote reference site clone (F1R9 = farm 1 reference site at 9 months, F2R9 = farm 2 reference site at 9 months). ** indicates the 9 month sampling period.

Figure 8. 16S rDNA similarity tree showing positions of members of the all remaining bacterial groups found within the marine sediments in this study. Red type denotes farm 2 (F2C9 = farm 2, cage site at 9 months, F2C12 = farm 2, cage site at 12 months), blue type denotes Farm 1 (F1C9 = farm 1, cage site at 9 months, F1C12 = farm 1, cage site at 12 months) and green type denote reference site clone (F1R9 = farm 1 reference site at 9 months, F2R9 = farm 2 reference site at 9 months). ** indicates the 9 month sampling period.



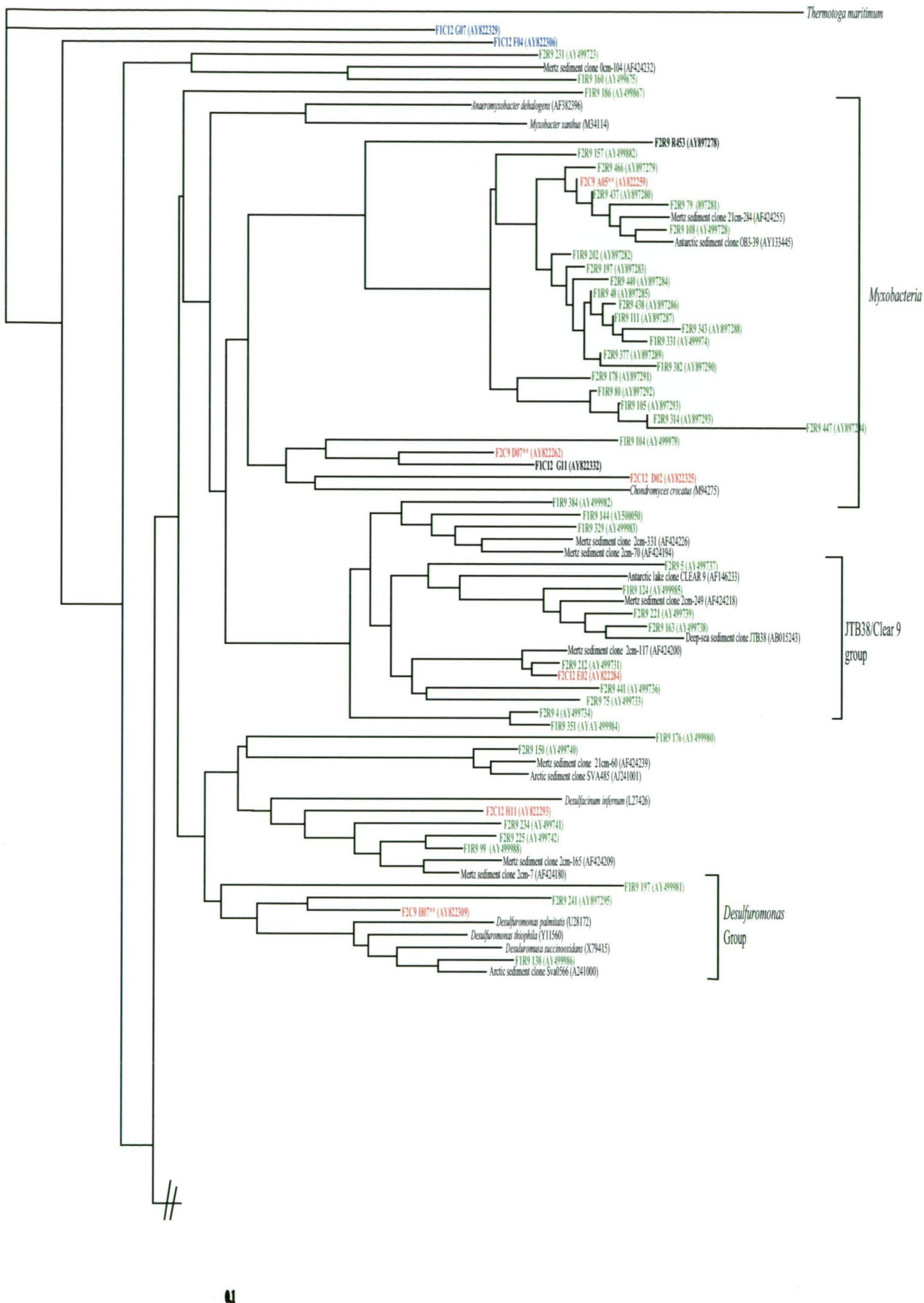
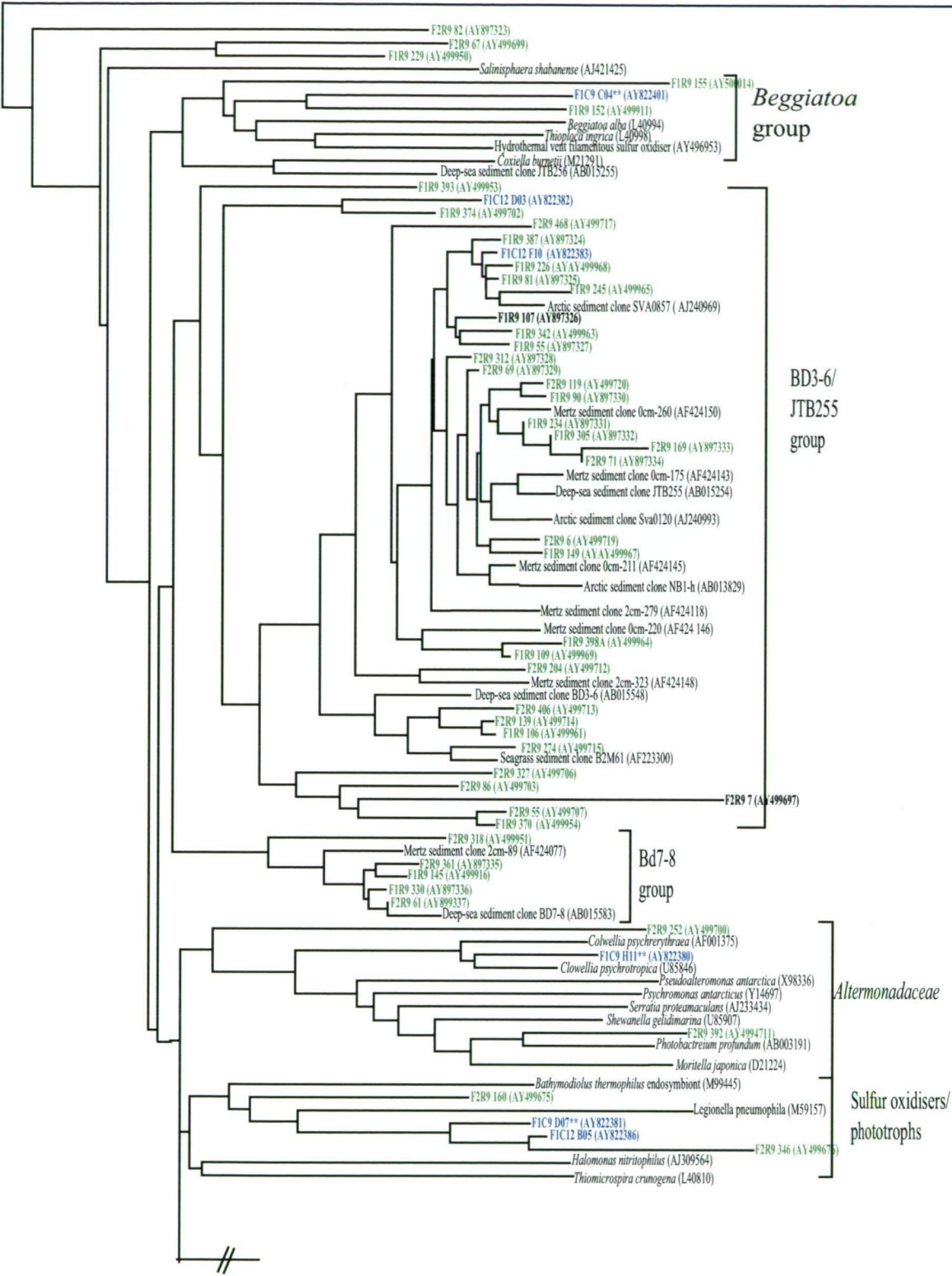


Figure 3a.

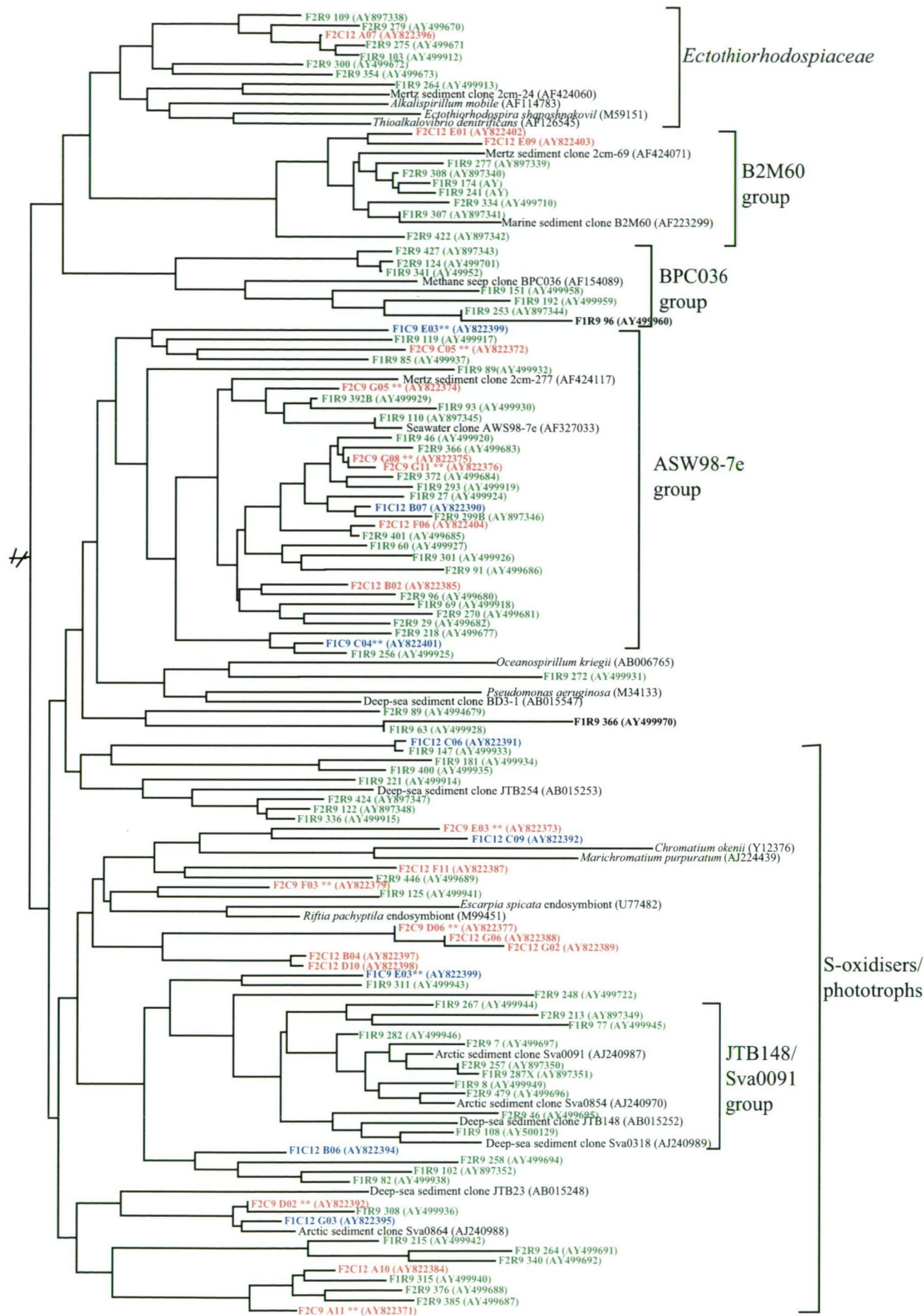


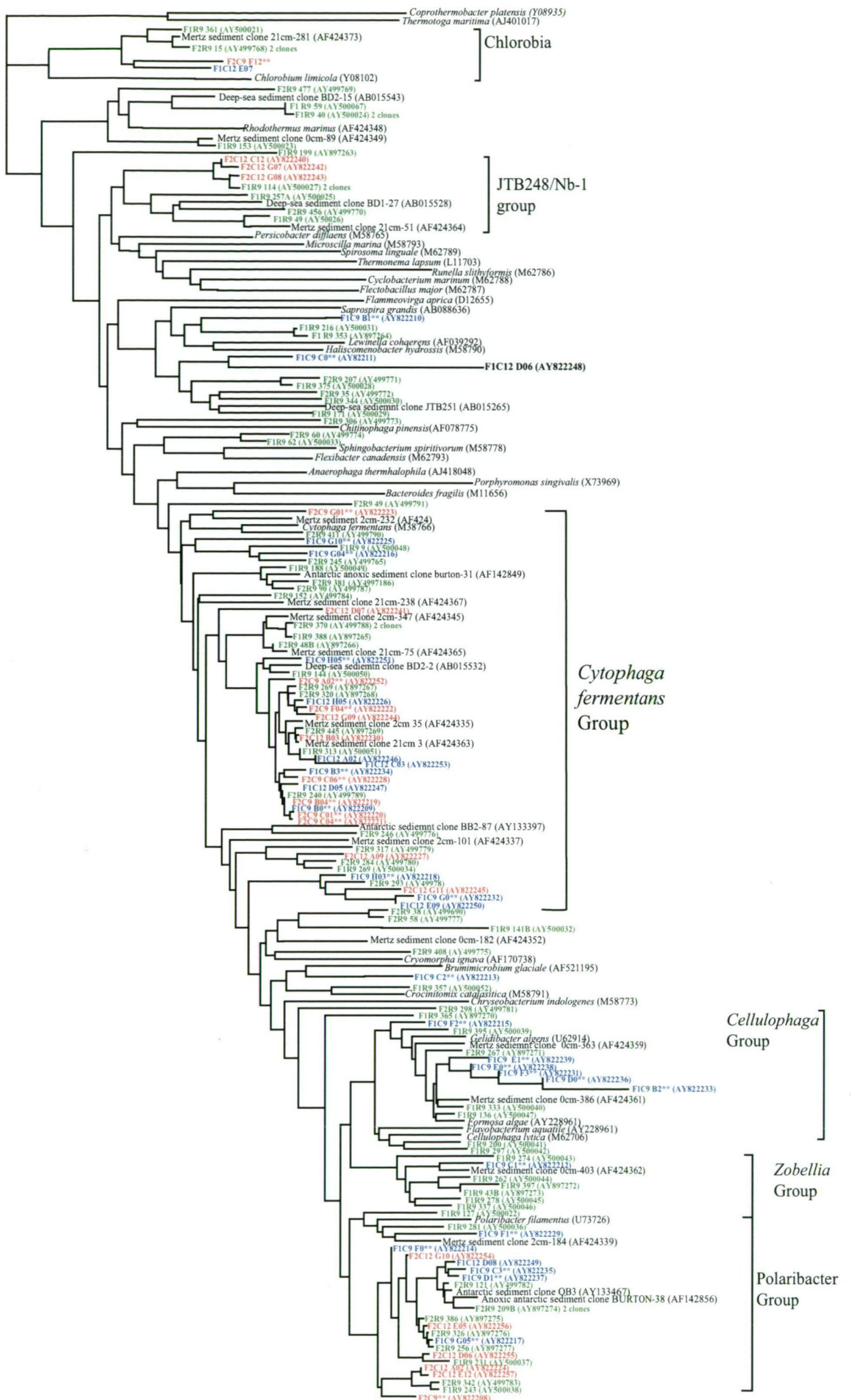
Figure 3b.

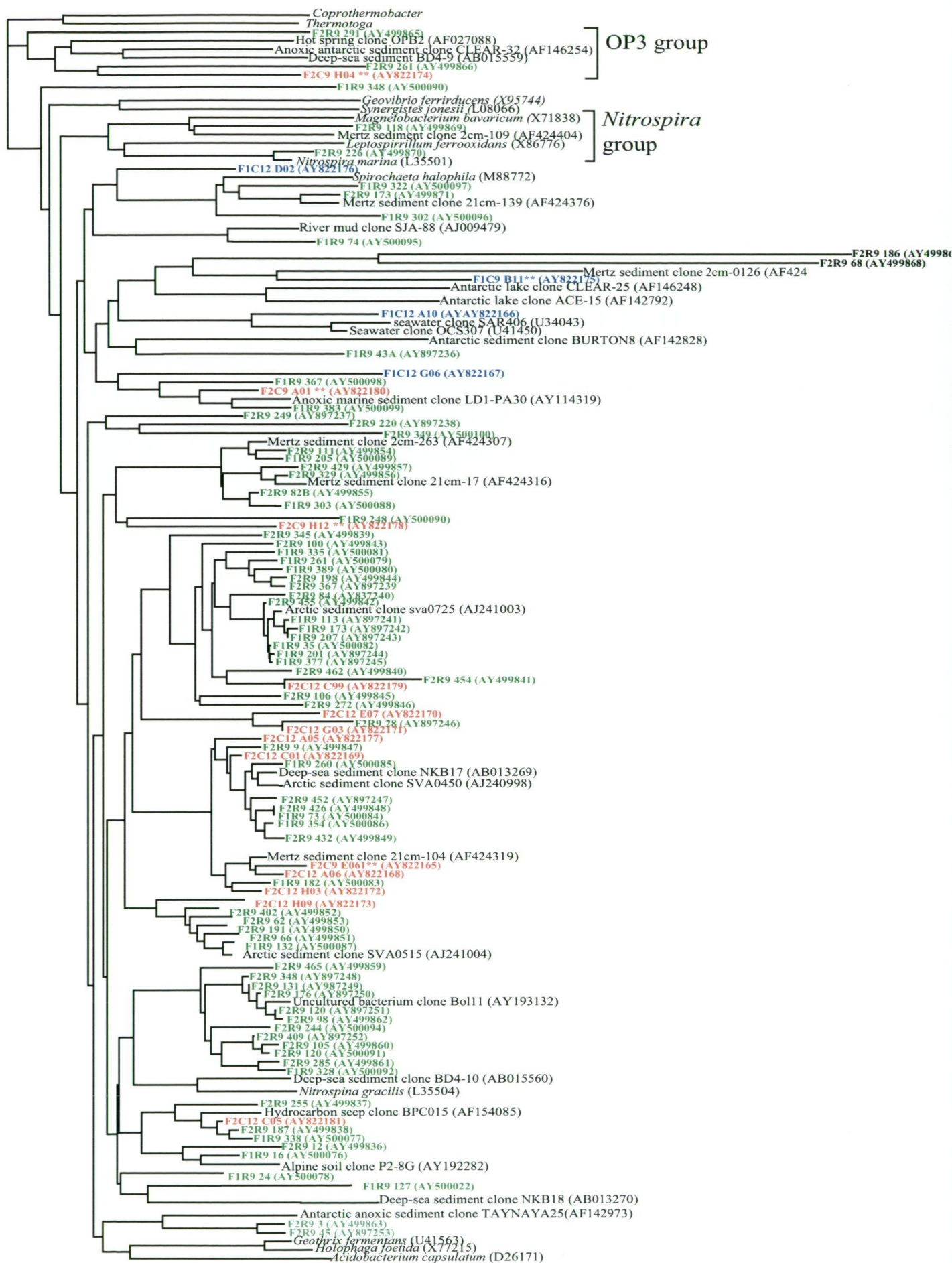


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Figure 4a.

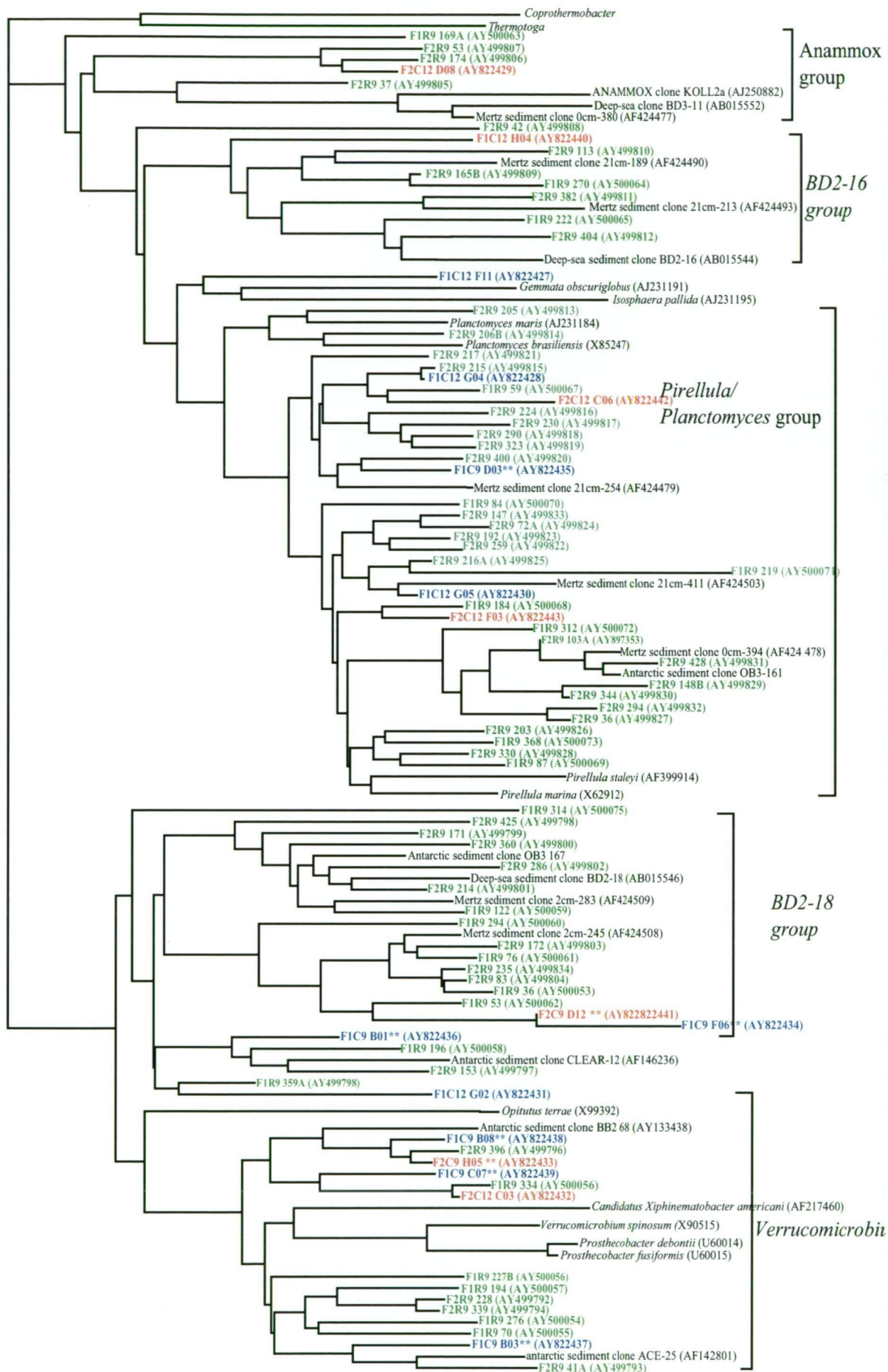






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Figure 6.



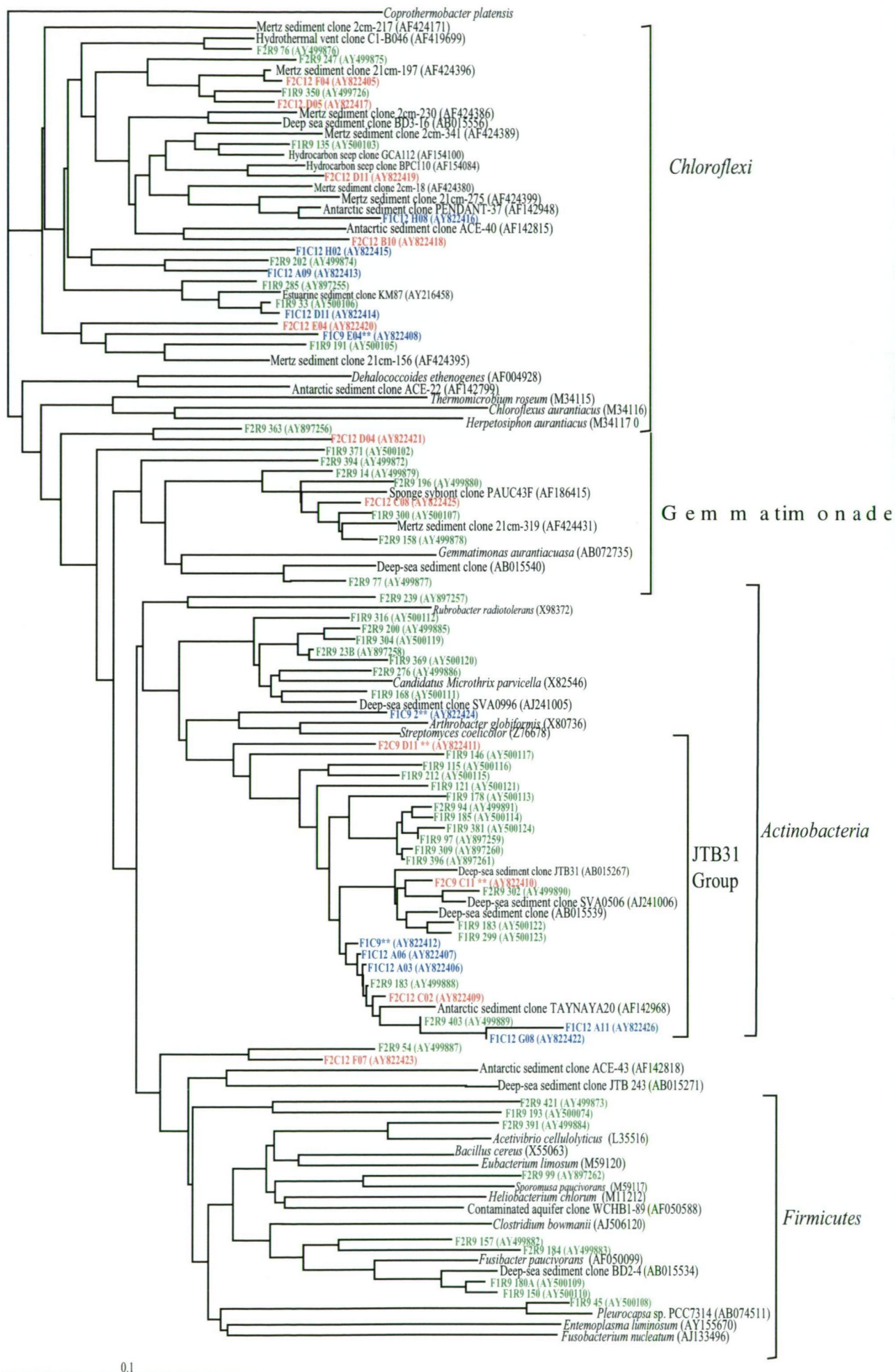


Figure 8.

3.3.3 Clone library comparisons

The hypotheses that the cage site libraries would not be significantly different from the reference site libraries at each farm and that the cage site libraries from each farm would not be significantly different at varying times during the production cycle were tested using by the LIBSHUFF method (Singleton, Furlong, Rathbun, Whitman, 2001).

In all comparisons both the heterologous and homologous coverages were similar (Table 3- 1). In all comparisons except comparison 1, the compared libraries were significantly different from one another (Table 3- 1). In comparison 1, however, the farm 1 libraries at the end of the stocking period and at the end of the fallowing period were not significantly different from one another (Table 3- 1). Additionally, comparisons of the calculated value of $(C_X - C_{XY})^2$ to the 95% value of $(C_X - C_{XY})^2$ from the random shuffles showed that differences between the libraries were greatest at distances ≤ 0.08 (data not shown). This suggests that differences that did occur were mostly among closely related sequences, a result that could be expected given the low coverage at high levels of relatedness. This is supported by the similarity trees (Figures 2 - 8) in which sequences from all samples and sampling times often grouped closely together, but were seldom identical.

Table 3-1 LIBSHUFF comparisons of sediment clone libraries. Bon-ferroni corrected $\alpha = 0.0085$.

Comparison	Homologous coverage data	Heterologous coverage data	<i>P</i>
Number			
1	F1C9	F1C12	0.357
	F1C12	F1C9	0.310
2	F2C9	F2C12	0.003
	F2C12	F2C9	0.001
3	F1C9	F1R9	0.001
	F1R9	F1C9	0.001
4	F1C12	F1R9	0.001
	F1R9	F1C12	0.002
5	F2C9	F2R9	0.001
	F2R9	F2C9	0.001
6	F2C12	F2R9	0.001
	F2R9	F2C12	0.008

3.3.4 Diversity Indices

Several diversity indices were calculated for all libraries. The indices calculated approached the maximum for all libraries, but for cage sites were generally higher at the end of the fallow period and were highest at the reference sites (Table 3- 2). The coverage was low for all libraries, which would be expected for extremely diverse communities. Because the library sizes were different rarefaction was used to compare sequence richness within each library at standardised sample sizes (Figure 3- 2). At Farm 1 the chao1 estimate does not level out for any of the samples, but richness is seen to be greatest at the reference sites (Figure 3- 2). At Farm 2 the Chao1 estimate again does not approach an asymptote for any of the samples, although the estimates for the cage site at nine months are lower than for the other two samples (Figure 3- 2B). The reference site libraries were considerably larger than the cage site libraries, but still the chao1 estimator did not level off as more clones were

sampled (Figure 3- 2C). These results indicate that all sediments sampled were extremely diverse and the actual bacterial diversity in these sediments is a lot greater than was sampled in this study.

Table 3-2 Clone library summary information and diversity/richness measures for clones libraries generated from farms 1 and 2.

Sample	Unique species	Clones Sampled	Margalef richness (d)	Shannon Index (H')	Simpson's 1- λ	Chao-1 Estimator
F1C9	38	52	9.364	3.462	0.9789	294.0 \pm 93.97
F1C12	57	77	12.89	3.771	0.9747	490.5 \pm 126.2
F2C9	39	73	8.857	3.41	0.9642	249.25 \pm 80.91
F2C12	65	82	14.52	4.07	0.9922	273.28 \pm 58.4
F1R9	207	303	36.05	4.981	0.9910	1467.04 \pm 193.9
F2R9	225	316	38.92	4.143	0.9939	2165 \pm 288.6

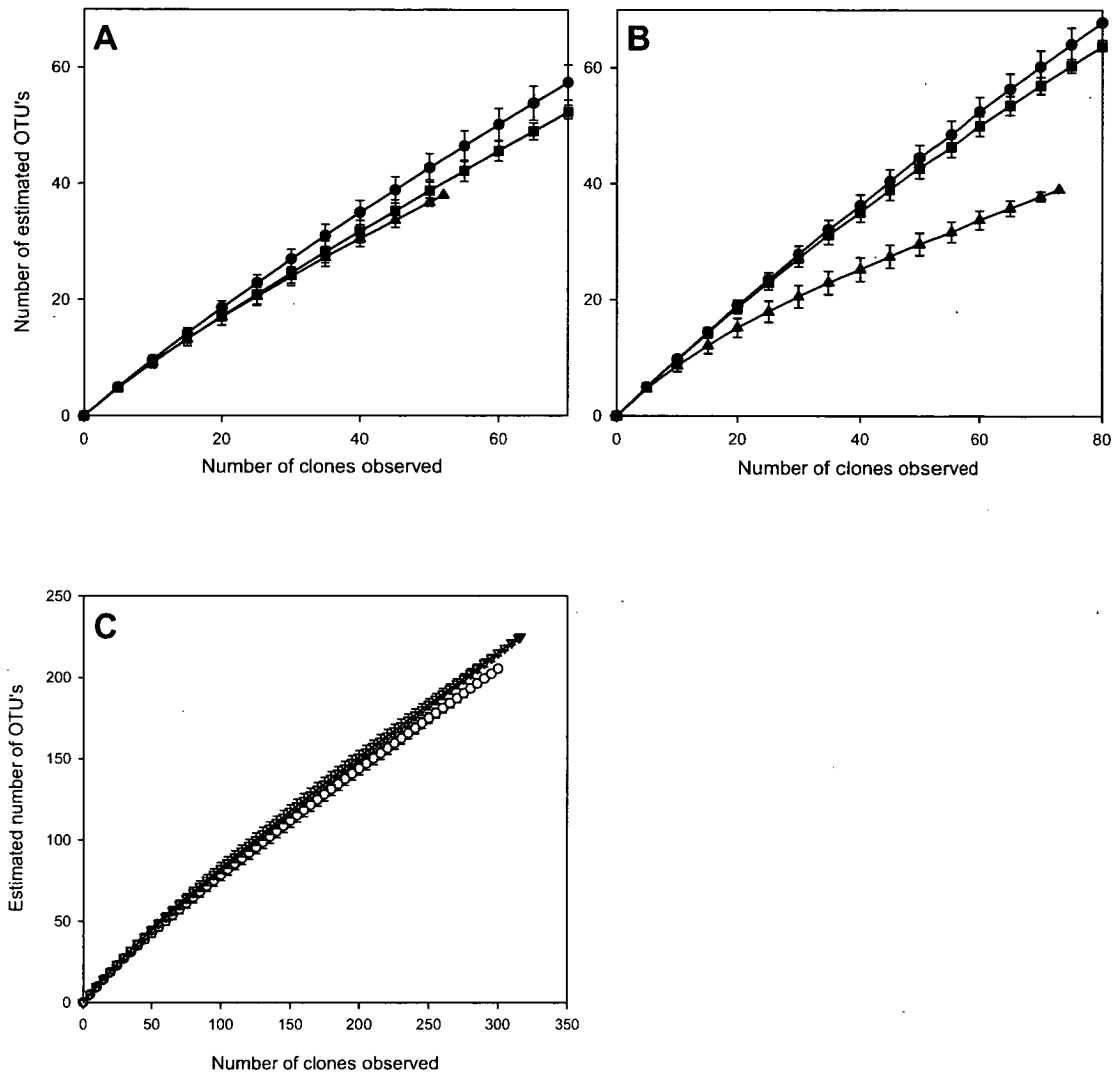


Figure 3-2 Chao1 estimates of bacterial richness of cage sites at nine months (▲) and twelve months (■) and at reference sites (●) at A) farm 1 and B) Farm 2. C) Chao1 estimates for reference sites at Farm 1 (○) and Farm 2 (▼). Error bars represent 95% CI.

3.4 Discussion

The clone libraries described here are the first constructed for Tasmanian coastal and aquaculture sediments. As such they provide an important insight into the microbial community diversity in these sediments and the potential effects of organic loading on this diversity. It should be noted, though, that clone library construction is very resource intensive and for this reason the replication and clone generation are

minimal. That is, libraries were constructed in an attempt to explore resident bacterial phylotype diversity, rather than to assess the on going community shifts that may occur under conditions of changing organic load. As is discussed in chapter 1, clone library construction and analysis is not a practical method to assess long term changes in communities, but is useful to build a database of phylotypes present. For this reason most effort was directed at reference site libraries, in which over 300 clone sequences per site were generated (Table 3- 2). For cage site libraries it was hoped that any major departures in community structure from the reference sites would become apparent with less sampling effort, but it was understood that subtle community changes may be overlooked. Indeed, judging by coverage estimates (Table 3- 2, Figure 3- 2) even sampling 300+ clones per library may not have elucidated subtle community changes. Cage site libraries comprised between 50 and 100 clone sequences (Table 3- 2). It is also interesting to note that no beta-proteobacterial sequences were found in these sediments. Phylotypes within this group are responsible ammonia oxidation and are seen as important to the nitrification process in sediments. The beta-proteobacterial ammonia oxidisers are, though, slow growers and are present in relatively low numbers (see chapter 5). It is likely that much more sampling effort would be needed to explore the total diversity of the sediments, rather than that of the more numerous constituents.

The distribution of clones found in this study, and the apparent absence of some groups, may also be the result of the shortcomings of PCR based studies. These have been discussed in detail elsewhere e.g. (Wintzingerode, Gobel, Stackbrandt, 1997). Although the issues of excluding groups via PCR primer bias (Schmalenberger, Schwieger, Tebbe, 2001; Vlasov, Dymshits, Lavrik, 1998) and increasing diversity artificially via PCR generated microheterogeneity need to be considered, they are common to all PCR based studies. The large number of clones sampled reduces the chances of missing less-well amplified products and the use of rarefaction analyses over ranges of sequence similarities reduces the effects of introduced microheterogeneity. It is likely some species are missing due to mispriming, but less likely that whole groups are excluded. Because groups such as the beta-proteobacterial AOB are present in relatively small numbers (see chapter 5) the chances of sampling such a group are diminished. The relative size of populations of

bacterial groups known to be important in geochemical cycling, such as the AOB, is more likely to contribute to their absence than PCR bias

Figure 3- 1 shows that there is some variation in the percentage composition of clone phylotypes between libraries. Clones were grouped into seven categories for this table, to simplify handling and analysis. The farm libraries did not exhibit a common pattern of community shift after cessation of organic load from farming. At farm 1 the library was dominated by the CFB group at the end of the farming period. During the three month following period the CFB group decreased as a proportion of the library, while the delta and alpha-proteobacteria appeared to take on more dominant roles. In the farm 1 reference library all groups were present, while at the farm 2 reference site no alpha-proteobacterial clones were found. Generally, the epsilon-proteobacteria were underrepresented at the reference sites when compared to the cage sites at both farms. This method of classifying clones into broad groups enables large differences in libraries to be viewed. These differences may not be observed if more resolution was applied and differences only looked for at 1-3% differences in sequences. The bacterial groups and phylotype composition are discussed in more detail below.

Diversity indices and statistical comparisons

The diversity indices presented in Table 3- 2 give insight into the diverse nature of the sediments under investigation. All of the indices used, except for the Simpson index, are sensitive to sampling effort (Clarke, Warwick, 2001). For this reason comparisons between similar sized libraries only are discussed when referring to these indices. At both sites there appears to be an increase in richness and diversity after the cessation of farming, as measured by the Margalef and Shannon indices and the Chao1 estimator. This increase is slightly more pronounced at Farm 2 (Table 3- 2). The reference sites appear similar in diversity as expressed by these measures. The Simpson's index suggests that all sediments were extremely diverse. This index, as expressed in Table 3- 2 ($1-\lambda$), may be thought of as an evenness index. While λ

expresses the probability that any two sequences chosen from the samples at random will be the same, $1-\lambda$ takes its greatest value when all species are distributed evenly. For all sediments the value of the Simpson's index approaches the maximum value of 1. This is even true for the reference libraries, which were subjected to considerable sampling effort. In a study of the genomic diversity in sediments Torsvik *et al* (1996) found that diversity in sediments under fish-farms decreased considerably, but increased after farming stopped. A similar pattern is observed in this study, but the differences are not as marked. This may be due to the different methods of assessing diversity. Torsvik *et al.* (1996) used a DNA rehybridization methods to assess the diversity of total bacterial DNA. Although the method employed by Torsvik *et al.* (1996) did not provide any phylogenetic information it was free of the artefacts of PCR (Wintzingerode, Gobel, Stackbrandt, 1997) and the constraints of the resource intensity necessary to adequately sample extremely diverse communities using clone libraries. Torsvik *et al.* (1996) report around 10,000 genomes in pristine marine sediments and approximately 60 and 1800 genomes in sediments under operational and abandoned fish-farms respectively. The richness estimates in this study (Table 3-2) indicate between 200 and 500 species are present at cage sites and approximately between 1500 and 2500 species at reference sites. Other studies of marine sediments have indicated similar richness calculations in pristine sediments (Bowman, McCuaig, 2003). The Chao1 richness indicator is, though, sensitive to sampling effort and should be interpreted with some caution. The reference sites may exhibit (and to some extent do) greater richness because they were subjected to greater sampling effort. The rarefaction analysis (Figure 3- 2) gives some insight into this. Figure 3-2a shows that no coverage curves for farm 1 approach an asymptote at the sampling effort given to cage sites. Figure 3- 2b shows similar results except that the F2C9 library coverage curve is less steep and may be approaching an asymptote. When the coverage curves are extended to include the sampling effort at the reference sites they still do not plateau (Figure 3- 2c). These data, and the fact that all the coverage curves except that for F2C9 are similar, suggests that the true diversity at these sites was not well sampled and that richness estimates are probably underestimates of the true diversity. It is likely that the clone libraries sampled the most abundant phylotypes and underestimated the rarer ones. One example of this is the already mentioned beta-proteobacterial phylotypes investigated in chapter 5, which do not appear in the clone libraries. The true diversity of all sediments is probably

somewhere between that indicated by the richness estimates in Table 3- 2 and that proposed by Torsvik *et al.* (1996). Finally the estimates for cage site richness in this study are greater than those indicated by Torsvik *et al.* (1996). There may be several reasons for this. Firstly, the impact of organic loading on Tasmanian aquaculture sediments is not as great as that on aquaculture sediments studied in much of the northern hemisphere (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). For example Brooks *et al.* (Brooks, Stierns, Backman, 2004) report sediment sulphide concentrations much higher and redox potentials much lower than were observed in the sediments studied here (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). The reasons for this difference probably lie largely in different farming intensity and farm practices in the various regions. Secondly, rehybridization rates of total sediment DNA may appear faster if the community becomes dominated by a single or few species (such as an opportunistic species), but still harbours much underlying diversity.

Libraries were also compared directly to one another using the LIBSHUFF method of Singleton (2001) and rarefaction (Table 3- 1 and Figure 3- 2). The LIBSHUFF comparisons showed all libraries except F1C9 and the F1C12 were significantly different (Table 3- 1). Differences in clone libraries may have been caused by differences in sediment types between the farms and also by differences in organic loading experienced at each site. Both heterologous and homologous coverage curves showed similar results, which is a little surprising. Because the reference libraries were much larger than the cage libraries it was expected that the cage site libraries would be shown to be a subset of the reference libraries. This was not the case, further supporting the idea that the diversity of these sediments was much greater than sampled here. All libraries were shown to be different, although inspection of the similarity trees reveals many similar phylotypes. The LIBSHUFF analysis looks at differences between the libraries at varying genetic distances (Singleton, Furlong, Rathbun, Whitman, 2001) and is able to give some insight into where the differences occur. In this study most differences occurred between 0.02 and 0.2 (data not shown) which indicates that differences in the library occur at both shallow and moderately deep phylogenetic groups. This is not entirely surprising. Figure 3- 1 indicates

differences in the libraries at large genetic distances and the large variation in 16S rDNA sequences at close distances is reported elsewhere (Bowman, McCuaig, 2003). Again it is testament to the great diversity in all the sediments sampled here that differences in the libraries were observed even though the cage site libraries only possessed the minimum number of clones as suggested necessary by Singleton (2001).

Sequence diversity: phylogenetic comparisons

When considering the phylotypes present it should be noted that the reference site libraries are much larger than the cage site libraries. The absence of phylotypes from either of the cage libraries and the relative number of clones in each group do not necessarily indicate the absence or dominance of certain phylotypes.

Phylotypes that fall in the alpha-proteobacteria group are present in the highest proportion in the F1 and F2C9 libraries and are absent from the F2C12 and F2R9 libraries (Figures 1 and 3). Most of the clones are associated with the *Roseobacter/Rhodobacter* group (Figure 3). This group is often associated with water column production and marine snow (Rath, Wu, Herndl, Delong, 1998). It is therefore not surprising to see this phylotype in the surface sediments of near-shore coastal waters, but the absence of clones in the F2C12 and F2R9 libraries is curious. Sedimentation and marine snow are more prevalent around salmon farms (Foster, 1996) and the fine sediment type at farm 2 suggests that it is in a deposition zone (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). It may be expected then that this group would be prevalent at farm 2 as well as farm 1. The answer to this curiosity may lie in the nature of the sedimenting material and the two farm environments generally. Farm 1 is more oceanic and exposed, farm 2 more sheltered and prone to terrestrial input, including fine material from freshwater systems. Further investigation is needed to answer the determinants of alpha-proteobacterial diversity in these systems.

The cage samples also dominated the epsilon-proteobacterial group (Figures 1 and 3) and all clones grouped into a single cluster. This cluster contains no representatives of cultured species, but does contain sequences found in a range of sediment habitats including anoxic and deep-sea sediments and hydrothermal vents (Figure 3). Little is

known about his group, but it has been referred to in a previous study as the “epsilon symbiont relatives” group (Madrid, Taylor, Scranton, Chistoserdov, 2001). This study suggested that the group were sulphide oxidisers that may be ectosymbionts of eukaryotic organisms. The eukaryotic fauna in these sediments was very sensitive to organic loading from the farms and the fact that this group of clones are more closely associated with the farm sites, and with the C9 libraries in particular, may suggest that this phylotype move into the sediments with a eukaryotic species. Further work is needed to confirm this though.

The delta-proteobacterial group were well represented in all libraries (Figures 1, 4a and 4b). The two largest groups were the *Desulfobulbaceae* and *Desulfosarcina* groups. Bacteria within these groups are known to be sulphate reducers and it is likely that many of the clones found in the sediments associated with these groups carried out this function. The sediments were reduced and showed visible signs of sulphate reduction (black sediments smelling of hydrogen sulphide). Several clones also fell in the *Desulfuromonas* group. This group contains phylotypes known to be capable of the reduction of elemental sulphur and iron and would be expected in the sediments under investigation. Clonal sequences associating with the Myxobacteria were also found in all libraries. It has been suggested that the Myxobacteria are not able to grow in marine conditions, but are found in clone libraries near shore from marine areas because they are washed into these areas from soils and freshwater systems and are able to adopt a resistant vegetative stage (Reichenbach, Dworkin, 2001). The fact that sequences from this group are evident in a wide range of marine sediments ((Bowman, McCuaig, 2003; Powell, Bowman, Snape, Stark, 2003) suggests that this may not be the case. It is highly likely that representatives of the Myxobacteria group are resident in these sediments, but have not been culturable. Phylotypes associating with groups not represented by any cultured organisms (JTB38 and Eel-TE1A4) comprise the remaining phylotypes (Figures 4a and 4b). Sequences in these groups have been associated with variety of marine deep sea, coastal and methane sediments.

The gamma-proteobacteria constituted a large proportion of all libraries (Figure 3- 1) and represented a very diverse group (Figures 4a and 4b). The gamma-proteobacteria contains phylotypes that, while being phylogenetically closely related, are often

phenotypically distinct from one another (Gray, Herwig, 1996). This fact should be considered when attempting to attribute possible phenotypes to environmental clones from this group. The majority of clone sequences fell in groups that were distinct from cultured species, but which have been observed in marine sediment environments previously. The clones found in the BD3-6/JTB255 group (Figure 4a) were almost entirely from reference libraries; only a single F1C12 clone being the exception. The same may be said for those clones falling in the JTB148/Sva0091 cluster. Interestingly, though, many cage site clones grouped with sulphur oxidizers/phototrophs either side of the JTB148/Sva0091 group (Figure 4b). The BPC036 group also contained only clones from the reference libraries. The reason for this may be either that the clones were rare/absent from the cage sites or that the increased sampling intensity at the reference sites has facilitated discovery of these groups in the reference sites. It may be possible that the latter explanation is the more likely, given that all groups are relatively small in this study and have clones from both reference libraries even though both sites possessed fairly different sediment characteristics. The *Beggiatoa* group is conspicuous by the paucity of sequences it contains (Figure 4a). The sediments under investigation (especially those at cage sites) were observed to possess mats of filamentous bacteria similar to *Beggiatoa* (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004), yet there are almost no *Beggiatoa* sequences in the libraries. Indeed there are no *Beggiatoa* sequences from farm 2, at which *Beggiatoa* were observed more frequently than at farm 1. This result may be an artefact of the sampling procedure which involved freezing the sediments prior to DNA extraction. Large cell types were seen to be more fragile when bacterial dispersal was attempted (see chapter 2) and it is possible that freezing lysed all *Beggiatoa* cells whose DNA was then lost to the sediment bulk. Alternately it is possible that even though the *Beggiatoa* were present in high enough numbers to be visible with the naked eye the large cells may possess only small amounts of chromosomal DNA. The result being that although apparently numerous, in terms of 16S rDNA contribution they comprise relatively little of the total bacterial DNA. The latter seems likely and the contribution of this group to sulphur, and perhaps nitrogen, cycling in the sediments should not be overlooked.

The CFB group bacteria were also well represented in all libraries, but were more dominant in the F1C9 library. This group showed a great deal of diversity (Figure 5)

and although the reference site clones were spread throughout all groups, the cage site clones were dominated by four clusters: the *Cytophaga fermentans* group, the *Cellulophaga* group, the JTB248/Nb-1 group and the *Polaribacter/Tenacibaculum* group. The clones from the cage libraries at farm 1 were distributed throughout all four of these clusters, while the F2C9 CFB clones were mainly restricted to the *C. fermentans* group. This group are predominantly anaerobic or facultatively aerobic and may represent an opportunistic phylotype taking advantage of the organic loading from the farm and continuing to grow in the anaerobic conditions generated. The *Cellulophaga* group, on the other hand, are predominantly aerobic. Clones from farm 2 cage libraries were absent from this group. The *Polaribacter/Tenacibaculum* group are also predominantly aerobic, and although the F2C12 library contains clones from this group, only one a single F2C9 clone from this group was seen.

The remaining bacterial groups are shown in Figures 7 – 9. Clones from these groups were predominantly from the reference libraries, although clones from the cage libraries are also seen throughout the trees. The distribution of clones and the predominance of reference site clones probably reflect the relative sampling effort of the libraries and the rarity of these phylotypes. It is interesting to note the presence of several clones within the *Planctomycetales* that are distantly associated with the ANAMMOX group (Figure 3- 7). ANAMMOX bacteria are responsible for the anaerobic oxidation of ammonia and may be important in the function of the coupled nitrification/denitrification process in anaerobic sediments. The presence of these clones suggests they are reasonably common (especially in the F2C12 library) and further investigation utilising analyses targeted to these organisms would be beneficial in understanding nitrogen cycling in sediments receiving organic loading.

3.4 Conclusion

Clone library analysis allows a detailed investigation of the phylotypes present in microbial communities that is not possible with fingerprint analyses. While library comparisons are influenced by sampling effort and the massive diversity of sediment communities will probably always suggest more sampling should have been undertaken, clone library construction provides much useful information from which more targeted research can planned.

In the sediments under investigation in this study it is evident that a large amount of bacterial diversity, both phylogenetic and phenotypic, is present. Many of the phylotypes present are those expected to be found in marine sediments and the majority are only described with reference to environmental sequences from other studies, but not with reference to cultured organisms. Sediment condition changed throughout the study, as organic loading and its cessation occurred (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004), and libraries were shown to be different from one another. This result suggests that all sediments possessed a large range of functional redundancy that contributed to their ability to maintain diverse communities despite the perturbation caused by increased organic load.

Chapter Four

Sediment Bacterial community shifts at two salmon farms

Chapter 4 Bacterial Community Shifts at two Salmon Farms

4.1 Introduction

Eutrophication of coastal waters is seen as one of the most important pollution issues facing marine ecosystems (Paerl, 1998; Savage, Elmgren, Larsson, 2002). The problem is exacerbated by increasing populations in coastal areas and increases in the intensity of industries that discharge organic pollutants to coastal areas. Aquaculture is one such industry. It has been widely reported (Findlay, Watling, Mayer, 1995; Macleod, Crawford, Moltschaniwskyj, 2004; McGhie, Crawford, Mitchell, O'Brien, 2000; Wildish, Hargrave, Pohle, 2001; Wu, 1995) that organic loading from fish-farms causes alterations to marine benthic communities; although this impact is usually restricted to the immediate area of the farm. It has also been suggested that anthropogenic organic input may have a more widespread effect on ecosystem function and may contribute to the change in system trophic status (Paerl, Dyble, Maoisander, Noble, Piehler, Pinckney, Steppe, Twomey, Valdes, 2003). The microbial response to perturbation, including organic loading, is rapid (Giller, Witter, McGrath, 1998; Paerl, Dyble, Maoisander, Noble, Piehler, Pinckney, Steppe, Twomey, Valdes, 2003; Rysgaard, Thamdrup, Risgaardpetersen, Fossing, Berg, Bondo Christensen, Dalsgaard, 1998). This rapid response makes the microbial community a prime candidate for the monitoring of marine ecosystem response to perturbation.

The interactions between ecosystem diversity, structure and function and the effect these factors have on ecosystem stability are currently poorly understood for sediment microbial communities. Two opposing thoughts have evolved regarding the impact of diversity on ecosystem function and stability. One idea expounds that increased species diversity improves both function and stability (Naeem, Thompson, Lawler, Lawton, Woodfin, 1995; Tilman, 1996), while the other maintains that it is the functional role of the organisms present that determines ecosystem function and stability, rather than the diversity of individual species present (Hooper, Vitousek, 1997). These ideas regarding diversity and its effect on ecosystem function have developed using terrestrial, macro-eukaryotic models and have been poorly investigated in microbial communities.

The diversity of sediment microbial communities is massive (Torsvik, Sorheim, Goksoyr, 1996) and it is likely that this diversity leads to a high degree of functional redundancy among micro-organisms. Bacteria have rapid growth rates, are able to rapidly fill expanding niches in changing environments and individual species within different functional groups often have differing responses to changing environments. These properties of microbial communities may result in very stable systems, but the direct contribution of species diversity to maintaining system stability is largely unknown, although Müller *et al.* (2002) reported that system performance was altered when diversity decreased.

The present study assessed both the species diversity and function of sediment microbial communities in fish-farm sediments to elucidate the impact of organic enrichment on microbial community stability. A genetic fingerprint technique, denaturing gradient gel electrophoresis (DGGE), was used to assess species diversity and community response to disturbance and its cessation. The key function of sediment microbial communities is mineralization, which was assessed by measuring respiration parameters (Müller, Westergaard, Christensen, Sørensen, 2002). Community function after disturbance (organic loading from the fish-farm) was measured from CO₂ production following substrate addition. The resistance of the community to further disturbance was assessed by the application of heat treatment prior to respiration measurement (Müller, Westergaard, Christensen, Sørensen, 2002).

4.2 Methods

4.2.1 DNA extraction and purification

DNA was extracted from sediments as set out in chapter 3.2.2.

4.2.2 Polymerase Chain Reaction (PCR)

For DGGE, fragments of the 16S *r*RNA gene were enzymatically amplified using a MJ Research DNA Engine (PTC-200) thermocycler with the primers 907F (GGCAGTTAAGGAAACTCAA) (Lane, 1991) and 1392RC (CGCCCGCCGCGCCCCGCGCCCGGCCGCCCCCGCCCCACGGGCGGT

GTGTAC) (Ferris, Muyzer, Ward, 1996). A GC clamp was attached to the reverse primer in order to increase DGGE gel separation. Reactions were performed using Clontech's Advantage 2 Polymerase Kit in accordance with the manufacturer's recommendations: Reactions of 50 μ l contained 10 x Advantage Taq PCR 2 Reaction Buffer, 1 μ l of Advantage PCR 2 *Taq* DNA polymerase, 1 μ l of template DNA (0.1 – 0.5 ng/ μ l), 10 pmol of each primer, 1.25mM of each deoxyribonucleoside triphosphate. Thermal cycling was carried out with an initial denaturation step of 95°C for 4 min followed by 19 cycles of denaturation at 95°C for 1 min, annealing at 64°C for 1 min (decreasing by –0.5°C every cycle), and elongation at 72°C for 2 min and 10 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and elongation at 72°C for 2 min; cycling was completed by a final elongation step of 72°C for 4 min. The presence and size of the amplification products were determined by agarose (1%) gel electrophoresis of the reaction product and ethidium bromide staining.

4.2.3 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed using the D-code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, USA) according to the method for perpendicular gels in the Bio-Rad manual. Samples were initially analysed with a 20% - 80% denaturing gradient using an 8% acrylamide gel. Samples were then re-run using a gradient of 40% - 65% for better band separation. Approximately 25 μ l of PCR product (with 5 μ l of 5X DGGE gel loading buffer) was loaded and the gel run at 60°C for 16 h. Gels were cooled and stained for 20 min. using 10 μ l of 10000x Sybr-gold nucleic acid stain (Molecular Probes, Eugene, USA) in 100ml Tris-acetate EDTA (TAE) buffer (40mM Tris-acetate; 1mM disodium EDTA; pH 8). Gels were imaged using a UV transilluminator and digital images captured on a Kodak DC60 digital camera fitted with a deep yellow #15 filter (Tiffen, Hauppauge, NY, USA).

4.2.4 Analysis of DGGE fingerprints

Though it is possible for single DGGE bands to contain multiple sequence homologues, each DGGE gel band was considered to be a single operational taxonomic unit (OTU). Individual bands were defined by a visually discernable signal above the gel background. Images of DGGE gels were analysed by assigning

numbers to each of the bands present on the gel and then scoring each sample to define presence or absence of each band. Lanes/samples with no bands were excluded from the analysis. Scoring of gel banding-patterns resulted in a binary matrix containing presence/absence data for each set of samples. Statistical analysis was then performed on this matrix.

4.2.4.1 Multivariate statistical analysis

The multivariate approach used in this study was similar to that advocated by Clark and Warwick (2001), namely the following steps: 1) A visual representation of the community by hierarchical clustering, canonical analysis of principle coordinates (CAP) (Anderson, 2003d; Legendre, Anderson, 1999) and non-metric multidimensional scaling (nMDS). 2) discrimination of the samples using non-parametric multivariate analysis of variance (NPMANOVA) to test for significant interaction terms, followed by 1-way Analysis of similarity (ANOSIM) to investigate sources of significant differences if they were returned in the global test. Each of these steps will now be considered in more detail.

The techniques of ordination and classification seek to build a visual representation of sample groups within a data set. Essentially they attempt to depict variation between and within groups of multivariate data in reduced dimensions; usually two or three. Although both techniques often give comparable results they suffer from different sources of distortion (Bonuso, Newton, Brower, Ivany, 2002). Clustering is more sensitive to small scale variations, while ordination is more sensitive to large scale variation. Ordination is also more able to depict gradient type responses: something clustering is incapable of. The clustering method chosen was the unweighted pair group method (UPGM). For ordination nMDS and CAP were employed. Although multiple techniques were utilised to build a visual representation of the data, only the plot that best created this representation is shown in the results, unless the different methods produced inconsistent results, at which time all plots are shown and discussed. All analyses were performed on Bray-Curtis similarity distances.

After visual representation of communities, using clustering and ordination techniques, significance testing was undertaken to elucidate statistical differences

between groups. Since two factors may be responsible for observed differences in groups, NPMANOVA (Anderson, 2001a; b) was used to test for the significant interactions between the two factors. Although it is possible to discount interactions based on observation of the nMDS plot, such an approach was not deemed realistic with the complex nature of the dataset at hand. Significant results were investigated further by 1-way ANOSIM (on treatment/time combinations if there was a significant interaction, or on each factor if no interaction existed).

Analyses were conducted using the software Primer 5.2.4, CAP (Anderson, 2003d), NPMANOVA (Anderson, 2003a) (for balanced designs), XMATRIX (Anderson, 2003b) and DISTLM v.2 (Anderson, 2003c) (for unbalanced designs).

4.4.3 Sediment respiration parameters

The effect of sediment organic load on microbial respiration after the addition of substrates was assessed as a proxy for the sediment bacterial communities' ability to withstand further disturbance. The method used followed that of Müller *et al* (2002). Briefly, 2.5 g of sediment was added to 10 ml of sterile sea-water and flasks sealed, leaving a gas headspace of 3 ml. Half the samples were held at 50°C for 12 hours, the remainder at 15°C. After this 12 hour incubation a substrate (40 mg glucose + 11.4 mg $\text{NH}_4\text{NO}_3 \text{ g}^{-1}$ of wet sediment) was added to the sediment slurry and all samples incubated at 15°C. The headspace CO_2 was measured over the next 72+ hours on a gas chromatograph (Varian, aerograph 920) with a flame ionisation detector after separation on an Altech CTR 1 packed column. Helium was used as the carrier gas.

4.3 Results

Generally, bands discernable on DGGE gels (Figure 4-1) were obtained with the primer set employed and banding patterns generated were able to be reproduced (data not shown). The use of universal primers did though produce gel lanes with relatively high background smears. These smears are not unusual when analysing very complex communities, such as sediments, and may represent unresolved bands.

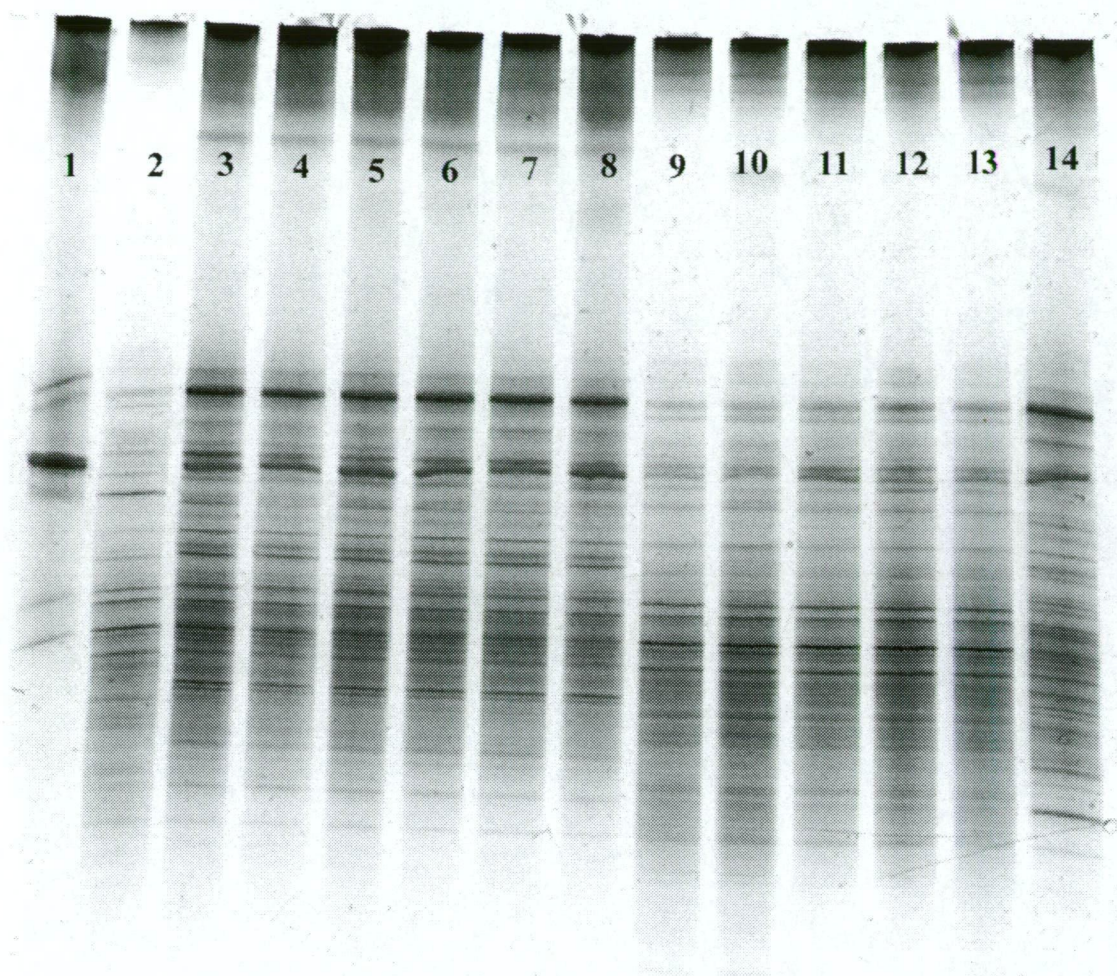


Figure 4-1 Inverted representative DGGE gel using universal primers 907F – 1392RC. Lane 1: PCR positive control. Lane 2: Farm 1, Cage 1, T0. Lane 3: Farm 1, Cage 1, T0. Lane 4: Farm 1, Reference 1, T2. Lane 5: Farm 1, Reference 1, T2. Lane 6: Farm 1, Reference 1, T2. Lane 7: Farm 1, Reference 2, T2. Lane 8: Farm 1, Reference 2, T2. Lane 9: Farm 1, Cage 1, T2. Lane 10: Farm 1, Cage 1, T2. Lane 10: Farm 1, Cage 2, T2. Lane 11: Farm 1, Cage 2, T2. Lane 12: Farm 1, Reference 1, T9. Lane 13: Farm 1, Reference 1, T9. Lane 14: Farm 1, Cage 2, T24.

4.3.1 DGGE diversity at farm 1

4.3.1.1 Stocking cycle 1

At farm 1 over the first stocking cycle there was a significant interaction ($F_{2,27} = 173.05$, $p = 0.0001$) between the effects of treatment and time on microbial community composition (Figure 4- 2A). Table 4- 1 lists planned ANOSIM results for treatment/time comparisons. Cage sites communities were significantly different from reference site communities at each sampling time. Reference site microbial communities shifted throughout the trial, to the extent that they were significantly different at each sampling time. The largest shift occurred between the nine and 12 month sampling periods (the fallowing period), with a smaller shift over the nine

month stocking period (reflected in the lower R-statistic value for this comparison).

Cage site communities were also different from one another at all sampling times.

The cage site microbial community at nine months, though, was not as variable as that at two or 12 months (Figure 4- 2A).

Table 4-1 ANOSIM results for farm 1, cycle 1 bacterial community fingerprints. T2 = 2 months, T9 = 9 months T12 = 12months of farm cycle 1.

Groups	R statistic	Significance (%)
Cage T2, Ref T2	1.0	0.8
Cage T9, Ref T9	1.0	0.2
Cage T12, Ref T12	1.0	0.2
Cage T2, Cage T9	1.0	0.5
Cage T2, Cage T12	1.0	0.5
Cage T9, Cage T12	1.0	0.2
Ref T2, Ref T9	0.58	1.5
Ref T2, Ref T12	1.0	0.2
Ref T9, Ref T12	0.922	0.2

4.3.1.2 Stocking cycle 2

During the second 12 month farm cycle at farm 1 the cage sites were not managed under the same time regime (see chapter 1.4), so each cage site and its complementary reference site is considered separately.

4.3.1.2.1 Cage1/reference 1

During the second farm cycle there was a significant interaction ($F_{2,12} = 105.49$, $p = 0.001$) between the effects of treatment and time on microbial communities at cage 1 and reference 1 (Figure 4- 2B). Table 4-2 presents ANOSIM planned comparisons for cage 1/reference 1 bacterial communities over the second farm cycle. As in the first 12 month cycle, cage communities were significantly different from those at reference sites at each sampling time. Reference site communities shifted during the nine month stocking period and again during the three month fallowing period. At cage sites the bacterial community appeared to undergo a large shift during the nine month stocking period, and then undergo a counter shift during the fallowing period. Reference sites also appeared to undergo a shift followed by a counter shift, but did not shift to the same extent that cage sites did.

Table 4-2 ANOSIM results for farm 1, cycle 2, cage1/reference 1 bacterial community fingerprints. T2 = 0 months, T9 = 9 months T12 = 12 months of farm cycle 2.

Groups	R Statistic	Significance (%)
Cage T12, ref T12	1.0	10
Cage T21, Ref T21	1.0	10
Cage T24, Ref T24	1.0	10
Cage T12, Cage T21	1.0	10
Cage T12, Cage T24	1.0	10
Cage T21, Cage T24	1.0	10
Ref T12, Ref T21	1.0	10
Ref T12, Ref T24	1.0	10
Ref T21, Ref T24	0.78	10

4.3.1.2.1 Cage2/reference 2

A significant interaction ($F_{2,12} = 19.74$, $p = 0.001$) also occurred between treatment and time at the second cage/reference pairing during farm cycle 2 at farm 1 (Figure 4-2C). Table 4-3 shows ANOSIM results for paired comparisons of treatment time combinations over the second farm cycle. Cage and reference sites always possessed bacterial communities that were significantly different from one another (even though $p>0.05$, see discussion). At both cage and reference sites it appeared that there was a smaller shift in community composition over the nine month stocking period, than over the subsequent three month following period. This is reflected in the R statistic values for the reference site comparisons, but not in the cage site comparison values, because of the small variation in samples at cages at the end of the nine month stocking period. The above interpretation is supported by cluster analysis (not shown).

Table 4-3 ANOSIM results for farm 1, cycle 2, cage2/reference 2 bacterial community fingerprints. T2 = 0 months, T9 = 9 months T12 = 12 months of farm cycle 2.

Groups	R Statistic	Significance (%)
Cage T2, Ref T2	1.0	10
Cage T9, Ref T9	1.0	10
Cage T12, Ref T12	1.0	10

Cage T2, Cage T9	1.0	10
Cage T2, Cage 12	1.0	10
Cage T9, Cage T12	1.0	10
Ref T2, ref T9	0.444	10
Ref T2, RefT12	0.907	10
Ref T9, Ref T12	0.926	10

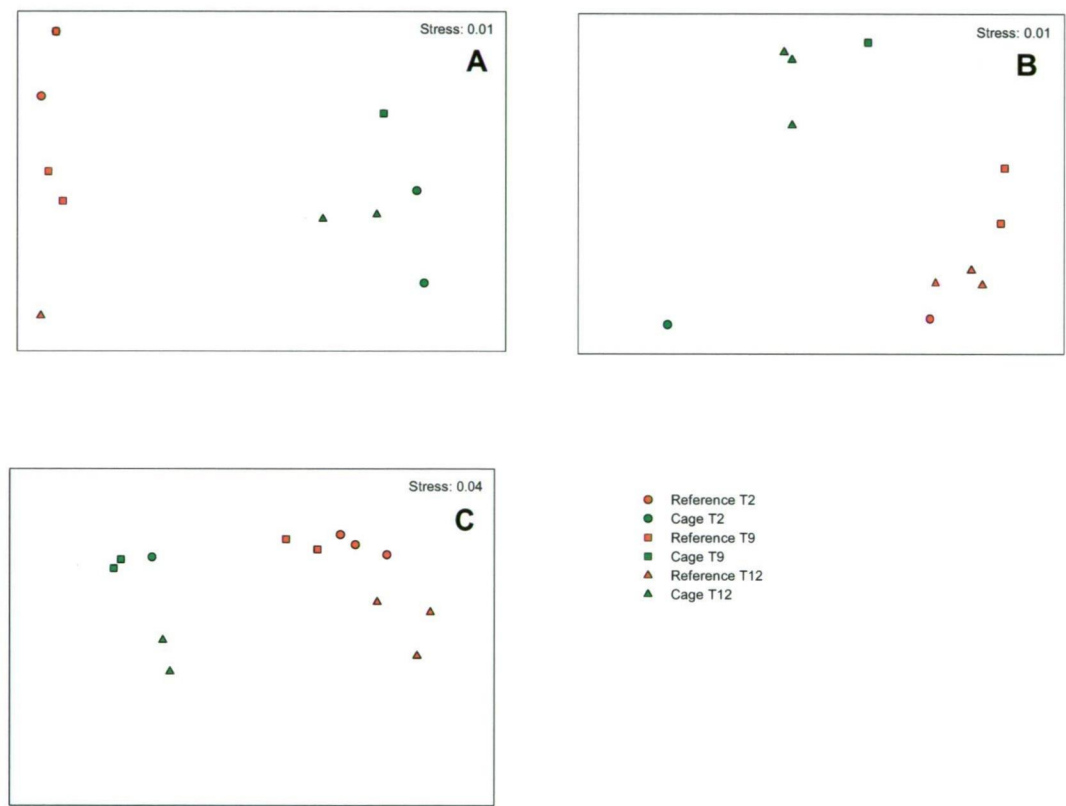


Figure 4-2 Farm 1 Ordination analysis – 2-dimensional nMDS plot of OTU presence/absence data from DGGE analysis, A) cycle 1, B) Cage 1 and Reference 1 during cycle 2 and C) Cage 2 and Reference 2 during cycle 2. T2 refers to the beginning of each farm cycle, T9 to the end of the stocking period and T12 to the end of the fallow period. N = 3.

4.3.2 DGGE diversity at farm 2

At farm 2 there was no significant interaction between the effects of treatment and time on microbial community composition, but both factors did affect community

composition in their own right (treatment $F_{1,26} = 7.06$, $p = 0.002$; Time $F_{2,26} = 3.16$, $p = 0.01$). Cage and reference site communities were always different from one another and communities at different times were always significantly different (Figure 4- 3, Table 4-4). These results were supported by cluster analysis. Reference site communities exhibited more variation than cage site communities.

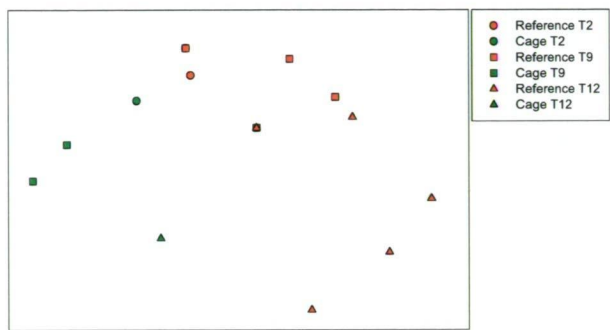


Figure 4-3 Farm 2, cycle 1 Ordination analysis – 2-dimensional CAP plot of OTU presence/absence data from DGGE analysis. T2 refers to the beginning of the farm cycle, T9 to the end of the stocking period and T12 to the end of the fallow period farm 2, cycle 1.

Table 4-4 ANOSIM results for the effect of time on microbial community composition at farm 2, cycle 1. T2 = two months, T9 = nine months and T12 = twelve months of farm cycle 1.

Groups	R Statistic	Significance (%)
T2, T9	0.822	0.1
T2, T12	0.810	0.1
T9, T12	0.809	0.2

During the second farm cycle at farm 2 both treatment ($F_{1,29} = 8.09$, $p = 0.001$) and time ($F_{2,29} = 2.87$, $p = 0.02$) had a significant effect on microbial community composition (Figure 4- 4). Table 4-5 presents ANOSIM results for comparisons of the effect of time. The bacterial community shifted at both cage and farm sites over the nine month stocking period and shifted further away from the community at the beginning of the trial during the 3 month fallowing period. The community shift during the fallowing period, though, wasn’t large enough to make this community significantly different from the community at the end of the stocking period.

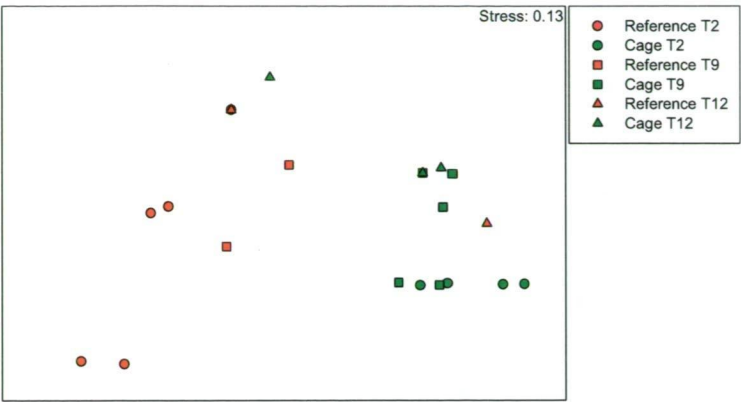


Figure 4-4 Farm 2, cycle 2 Ordination analysis – 2-dimensional nMDS plot of OTU presence/absence data from DGGE analysis. T2 refers to the beginning of the farm cycle, T9 to the end of the stocking period and T12 to the end of the fallow period farm 2, cycle 2. N = 3.

Table 4-5 ANOSIM results for the effect of time on microbial community composition at farm 2, cycle 1. T2 = zero months, T9 = nine months and T12 = twelve months of farm cycle 2.

Groups	R statistic	Significance (%)
T12, T21	0.501	0.1
T12, T24	0.756	0.1
T21, T24	0.078	28.7

Banding patterns from farm 2 were also compared for two cage sites over the 24 month trial (T2, T12, and T24) to assess the efficacy of a longer (15 month) fallowing period in mitigating the effects of organic loading from the farm. ANOSIM demonstrated a significant effect ($R = 0.894$, $p = 0.001$) of time on the microbial communities at cage sites over the trial. Communities were different from one another at all three times over the 24 month period (Table 4-6, Figure 4- 5). A shift in the community occurred between the beginning of the trial and the end of the first fallowing period, while a counter shift appeared to occur during the following 12 months, suggesting the microbial community was closer to it’s start point after 15 months fallowing than it was after 3 months fallowing.

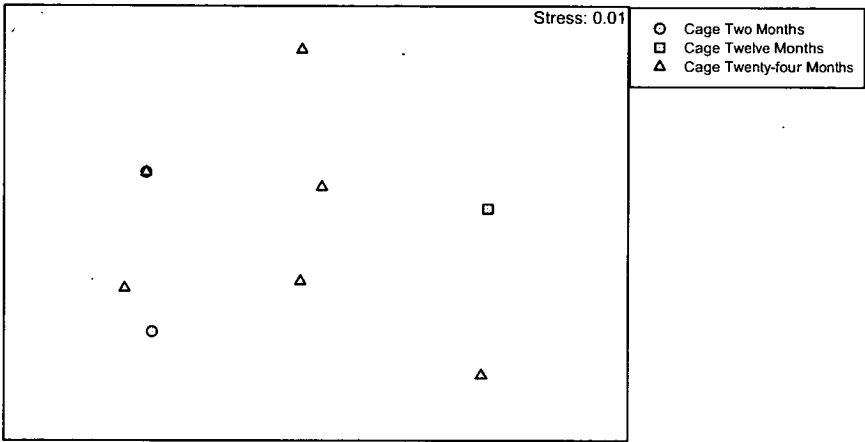


Figure 4-5 Farm 2, Ordination analysis – 2-dimensioal nMDS plot of OTU presence/absence data from DGGE analysis at farm 2 cage sites over the 24 month stocking cycle. N = 3.

Table 4-6 ANOSIM results for comparisons of cages at farm 2 over the 24 month trial. = two months, T12 = twelve months and T24 = twenty-four months.

Groups	R Statistic	Significance (%)
Cage T2, Cage T12	1.0	0.5
Cage T2, Cage T24	0.667	2.9
Cage T12, Cage T24	0.762	0.5

4.3.3 Community richness at farm 1 and 2

Community richness was measured as the number of bands (OTU) per lane for each sample. Tables 7 and 8 show OTU richness, over both farm cycles, at farm 1 and 2 respectively.

Table 4-7 Mean OTU richness (bands/lane) ± SE at Farm 1 over farm cycle 1 and 2. Superscripts refer to *post hoc* tests for each farm cycle (not between farm cycles). During cycle 2 *post hoc* tests were performed on each cage/reference pair. Samples sharing a common superscript are not significantly different (p>0.05).

	Farm Cycle 1		Farm Cycle 2			
Time	Cage	Reference	Cage 1	Reference 2	Cage 2	Reference 2
T2	17.5 ± 0.3 ^c	12.8 ± 0.2 ^b	13 ± 0 ^{ab}	12 ± 0 ^a	20 ± 0 ^c	11.7 ± 0.9 ^{ab}
T9	20 ± 0 ^d	13.5 ± 0.2 ^b	19 ± 0 ^d	15.3 ± 0.3 ^c	22.3 ± 0.3 ^c	14.7 ± 0.7 ^b
T12	13.2 ± 0.2 ^b	12 ± 0 ^a	14.7±0.7 ^{bc}	12.3 ± 0.7 ^a	14 ± 1.0 ^b	9.7 ± 1.2 ^a

At farm 1, richness is higher at cage sites at all times except between cage 1/reference 1 at the beginning of the second farm cycle. During both cycles richness at cages increased over stocking and decreases during fallowing. A similar pattern was exhibited at the reference sites, but changes were more subtle.

Table 4-8 Mean OTU richness (bands/lane) ± SE at Farm 2 over farm cycle 1 and 2. Superscripts refer to *post hoc* tests for farm cycle 1. No interaction effect was detected for cycle 2, so no *post hoc* comparisons were carried out. Samples sharing a common superscript are not significantly different (p>0.05).

	Farm Cycle 1		Farm Cycle 2	
Time	Cage	Reference	Cage	Reference
T2	13.0 ± 0 ^{bc}	10.3 ± 0.3 ^b	10.4 ± 0.4	4.5 ± 1.2
T9	14.2 ± 0.5 ^c	6.3 ± 1.9 ^a	12.0 ± 0.5	7.0 ± 0
T12	15.0 ± 0 ^c	5.4 ± 0.9 ^a	12.4 ± 0.9	5.9 ± 2.2

At farm 2, richness is again generally higher at the cage sites, but does not exhibit the same response to farming and fallowing observed at farm 1. In cycle 1, richness

stayed elevated after fallowing, while during cycle 2 richness was always higher at cage sites ($F_{1,35} = 21.0$, $p < 0.000$).

4.3.3 Respiration parameters at farm 1 and 2

Respiration parameters were measured after substrate amendment as % CO₂ in headspace gas (Müller, Westergaard, Christensen, Sørensen, 2002) at both farms at the end of the stocking and fallowing periods of farm cycle 2. Results are presented graphically in Figure 4- 6. At farm 1 the classic response curve as reported by Nordgren *et al.*, (1988) was not observed, but treatments responded similarly at both 9 and 12 months. There was no exponential phase in respiration rate at either time. The two heat shocked treatments exhibited lower respiration rates than those not heat shocked, regardless of organic loading. Cage site sediments exhibited the highest rates of respiration.

At farm 2 treatments also responded similarly at both times. The non-heat shocked cage sediment exhibited the highest respiration rate, followed by the heat-shocked cage and non-heat shocked reference sediments, which performed similarly. At both times the heat-shocked reference site sediment exhibited the slowest response to substrate addition.

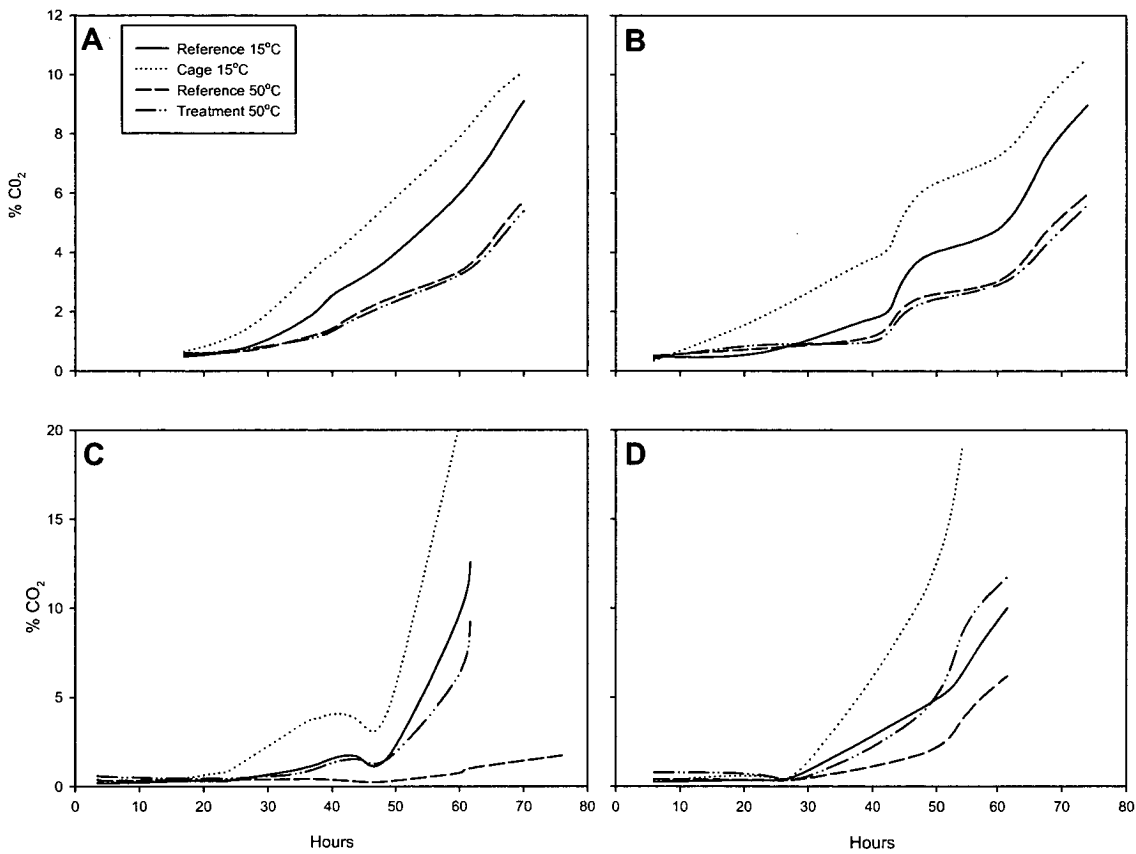


Figure 4-6 Percent CO₂ in headspace following substrate addition to sediments. A) Farm 1, end of stocking (9 months), B) Farm 1, end of fallowing (12 months), C) Farm 2, end of stocking (9 months), D) Farm 2, end of fallowing (12 months). Heat-shocked sediments are labelled as 50°C, non-heat-shocked sediments are labelled as 15°C.

4.4 Discussion

Sediment bacterial community shifts were monitored by DGGE at two salmon farms, over two, 12 month production cycles. DGGE does not provide an estimate of total bacterial diversity, but does provide a means to monitor shifts in the dominant community in a statistically valid manner (see chapter 7). The reproducibility of DGGE banding patterns has been investigated previously (Ferrari, Hollibaugh, 1999; Murray, Hollibaugh, Orrego, 1996; Powell, Bowman, Snape, Stark, 2003) and the sources of variation have been found to lie with both the PCR process and use of multiple gels. While it may be possible to use a single PCR run, as was done in this study, the utilisation of fingerprint techniques such as DGGE in ecological studies

will usually necessitate analysing more samples than can be run on a single gel. Indeed the ability to run large sample sets in a cost effective manner is the primary advantage of using fingerprint techniques. To assess the precision of the DGGE technique over multiple gels several samples were run on different gels prior to the analysis of the full sample sets (data not shown). This pilot-study demonstrated that although the banding pattern may be compressed or elongated the *gel signature* did not affect the interpretation of the banding patterns. This conclusion agrees with that of Ferrari and Hollibaugh (1999) who suggested that although a *gel signature* may be evident, the greatest difference in banding patterns results from differences in the samples themselves. In an attempt to account for the *gel signature* a single environmental sample was run in the outside lane of each gel. If the banding pattern of this control lane differed between gels (other than to be elongated or compressed) the gel was to be discarded and re-run. Such an event did not occur; all control banding patterns, and therefore presumably those of the remaining samples, were consistent between gels. It is suggested that a similar determination of the sources of variation in banding patterns be repeated for new studies, especially if it is not possible to run all PCR reactions in a single step.

The limitations of the multivariate significance testing techniques are also evident in the results (Tables 2 and 3). During the second stocking cycle at farm 1 the number of possible permutations was reduced because each cage was considered separately (the result of farm management changes discussed in chapter 1.4). As a result a significance level of 0.05 could never be achieved. Although a significance $p < 0.05$ could not be achieved it was still possible to assess differences between groups by analysing the R-statistics and the MDS plot. A large R value (close to 1) indicates near complete separation of the groups, while a small R value shows little separation. A large R value and an MDS plot of low stress that shows groups separate well substantially supports a finding of differences between groups, even though the test may not have enough power to demonstrate this with a $p < 0.05$. The R value is not affected by replicate number, unlike its statistical significance (Clarke, Warwick, 2001).

At farm 1 there was a significant combined effect of both the farm and time on the microbial community composition over both farm cycles, and cage and reference site

communities were different at all sampling times. The first sampling time was two months after the commencement of the stocking cycle and so was not a true time-zero sample. Rapid response of some sediment microbial communities to anthropogenic input has been documented previously (e.g. (Delille, Delille, Pelletier, 2002; Griffiths, Caldwell, Broich, Morita, 1981; Rossello Mora, Thamdrup, Schaefer, Weller, Amann, 1999)). The response of the microbial community in these sediments is also very rapid. Despite this rapid response the communities at cage and reference sites remain different after the three month fallowing period. During the first year of the study the bacterial community at the reference sites shifts continuously away from that observed at the first sampling time, while the cage site community exhibits a shift over the first nine months followed by a counter shift over the fallow period. During the second year both cage and reference sites exhibit shifts in similar directions, although they remain significantly different. It is evident that there is a seasonal component to the variation in community structure, but that the main source of differences between the cage and reference site communities is the effect of the farm.

At farm 2 there is no interaction in the effects of the farm and time, but both factors do significantly affect the community composition. Again cage and reference sites are always different and communities shifted between sampling times. As with the response at farm 1, it is evident that the farm has a large effect on community composition, but there is also a seasonal component. The fallowing period did not result in a return to the bacterial community at reference sites, but rather caused further shifts in the community composition.

Respiration parameters were measured after further perturbation as a proxy for sediment functional stability. The rationale for this measurement is that if diversity is positively correlated with stability, then a loss of diversity would lead to a system that may be functionally compromised by further perturbation. This technique has been shown to be a sensitive indicator of soil community response to heavy metal contamination (Müller, Westergaard, Christensen, Sørensen, 2002; Nordgren, Baath, Soderstrom, 1988). In my study a heat shock was applied to sediments, as a further disturbance, after organic loading from fish-farms. At farm 1 the heat shock produced sediments with lower respiration rates than those not heated. Both heated sediments (cage and reference) responded similarly. This result suggests that cage sediments

were at least as diverse (resistant) as those at reference sites. The same may be said for sediments at farm 2; at which reference sediments performed more poorly than cage sediments. It should be noted that factors such as microbial biomass also affect respiration rate. The higher rates shown by non-heated cage sediments may be explained by their higher biomass. Although this method is difficult to interpret on its own, it is useful in supporting the fingerprint diversity data.

It is interesting to note that throughout the trial the reference site bacterial communities exhibited more within-group variation. This variation is evident on the nMDS plots shown in chapter 4.3. Intuitively, it would be suspected that less variation would occur in communities at cage sites, as the environment becomes more extreme and diversity decreases. Although variation in banding patterns is less at cage sites, there does appear to be a concomitant decrease in diversity. The diversity of dominant phylotypes (as measured by DGGE band number) actually increases under conditions of organic loading at both farms, although this phenomenon is more pronounced at farm 1. The limits of using fingerprinting techniques to measure diversity/richness are discussed in Chapter 1, and should be considered when interpreting this result. There are several possible explanations to the increased richness at the cage sites. Instead of having a deleterious effect on bacterial diversity the relatively rapid changes that these sediments are subjected to (9 months loading, 3 months fallowing) may actually increase the number of bacterial types that are prevalent/dominant in the microbial community. The sediment environment under the farm changes rapidly and often enough to continually open new niches, which are filled randomly by the many suitable candidate species existing in these diverse sediments. This constant flux may result in higher diversity than if change was permanent. Another explanation for the increased OTU richness is that a zone of enhancement has been formed. Pearson and Rosenberg (1978) reported such zones as *ecotones*. Such a zone supports both the opportunistic species that take advantage of the organic loading, as well as the species that originally inhabited the zone. It is probably a combination of both factors that contribute to the increased richness. Opportunistic heterotrophs (for example the CFB) certainly appeared to take advantage of the organic loading from the farms (see chapter 6), but other species still appeared to remain, including those not favoured by the enriched conditions. Bioturbation, which became increasingly important as the cage sites became

dominated by *Capitella* spp. communities, may have resulted in the formation of oxic micro zones that provided refuge for some bacterial types. Sediment bacterial communities may not have received enough organic input (in terms of volume or time) to move into the classic polluted phase (Pearson, Rosenberg, 1978) of organic degradation, but appeared to remain in this transitory stage. The respiration data also support the assertion that microbial communities under cage sites were at least as diverse as those at reference sites. This assertion is further supported by the finding that the sediment communities appeared to process all farm inputs (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004); thus their assimilative capacity was not exceeded. Because the sediments in this study did not receive enough organic input, whether or not the microbial community would progress along the gradient proposed in infaunal studies (e.g. Pearson and Rosenberg, (1978)) remains to be elucidated.

The practice of fallowing did not return microbial communities to their pre-disturbance state, although a shift in the communities did occur with both short (3 month) and longer (15 month) term fallowing. OTU richness decreased with fallowing, indicating that opportunistic species may be in decline after the cessation of organic loading. Sediment microbial communities are very diverse and the functional roles of many bacteria are redundant. Bacteria also have rapid generation times and it is not clear how which organisms fill different niches is determined. Although the microbial community does not return to its pre-disturbance state in terms of the dominant organisms present (as measured with DGGE) the community does respond to changes in farm activity and organic loading and the number of OTU's detected does not decline with organic loading. Although the functional diversity of sediment microbial communities was not assessed, other measured parameters (e.g. redox, sulphide, benthic faunal communities) returned to near pre-trial conditions after extended periods of fallowing (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004), suggesting that similar niches may have been created after the cessation of loading to those existent before the trial. In such a scenario it is entirely possible that the bacterial population did return to its pre-trial state in terms of its functionality, even if it didn't in terms of the OTU's present.

4.5 Conclusion

The sediment microbial community was shown to respond rapidly to both the addition of organic material during farming and to its cessation during fallowing. The exact nature of the community shifts in terms of changes to diversity and function, both with the initial disturbance and during the recovery phase, are harder to elucidate. Although the community does not return to its pre-disturbance state, in terms of the major bacterial species present, it does maintain a high level of diversity at all times. Respiration parameter results, and the fact that the organic matter added is utilised, also suggest that the microbial community function maintains its integrity throughout the trial.

An understanding of the nature of the microbial community and its response to perturbation is of great importance to the understanding of system wide-effects of perturbation. The efficacy of using routine monitoring of the microbial community to track degradation and recovery of systems, though, is not so clear cut. The functional redundancy of many bacteria may preclude meeting recovery targets in terms of diversity shifts as measured by fingerprint techniques. This is discussed in more detail in chapter 7.

The sediment microbial community is highly diverse and appears to be in a constant state of flux. While it is difficult to monitor the microbial community in terms of the traditional before-after-control-impact concept it is evident that the community does not collapse after disruption by organic loading from these fish-farms and that the sediment environment does not become too extreme to support a diverse and active bacterial community.

Chapter 5

β -proteobacterial ammonia-oxidising bacteria in two salmon farm sediments

Chapter 5 Beta-Proteobacterial Ammonia-Oxidising Bacteria in Two Salmon Farm Sediments

5.1 Introduction

Cage culture of salmon leads to localized pollution of the underlying sediments by the accumulation of organic waste materials (uneaten food, faeces, etc.). A major constituent of salmon diets is protein and the outputs of farms are therefore high in nitrogen content.

The process of nitrification is an important process in the cycling of nitrogen in the environment. Denitrification prevents the build up of nitrogen, the major limiting nutrient for phytoplankton growth, via its expulsion from the system as N_2 gas (Blackburn, Blackburn, 1992). Oxidized nitrogen species are supplied to denitrifiers primarily by the process of nitrification. The main factors which affect nitrification rates are: ammonia concentrations, carbon dioxide and oxygen concentrations, temperature, salinity and pH (Kowalchuk, Bodelier, Heilig, Stephen, Laanbroek, 1998; McL.Macdonald, 1986). The conditions (low oxygen, reduced sediments, low pH, and high sulphide concentrations) associated with fish-farm sediments are therefore seen as inhibitory to the nitrification process.

The ammonia-oxidizing bacteria (AOB) carry out the first, rate-limiting step of nitrification (the oxidation of ammonia to nitrite) and fall into two monophyletic groups within the proteobacteria. The first contains the strains of *Nitrosococcus oceanus*, in the γ -subdivision proteobacteria, and the other contains the genera *Nitrosomonas* and *Nitrospira* in the β -subdivision of the proteobacteria (Kowalchuk, Stephen, De Boer, Prosser, Embley, Woldendorp, 1997).

Analysis of the structure of ammonia-oxidizing communities has been problematic because the AOB are very slow growing (colonies taking several months to form on solid media) and relatively difficult to isolate (McCaig, Embley, Prosser, 1994). Difficulties in utilization of traditional microbiological techniques to investigate natural microbial communities, particularly the ammonia oxidizers, have lead to the development and application of molecular techniques to identify and enumerate AOB communities (for example (McCaig, Embley, Prosser, 1994; McCaig, Phillips,

Stephen, Kowalchuk, Harvey, Herbert, Embley, Prosser, 1999; Purkhold, Wagner, Timmermann, Pommerening-Roser, Koops, 2003; Purkhold, Pommerening-Roser, Juretschko, Schmid, Koops, Wagner, 2000; Stephen, McCaig, Smith, Prosser, Embley, 1996; Stephen, Kowalchuk, Bruns, McCaig, Phillips, Embley, Prosser, 1998)). Denaturant gradient gel electrophoresis (DGGE) is one such technique used. DGGE is a good technique to track long term changes in microbial communities when large sampling effort is required (chapter 1.4.1).

Quantification of microbial communities is also an important aspect of microbial ecological studies, but the AOB are difficult to enumerate using traditional methods. They are usually present in relatively low numbers (Bruns, Stephen, Kowalchuk, Prosser, Paul, 1999; Hermansson, Lindgren, 2001; Mendum, Sockett, Hirsch, 1999) and are slow growing. Several methods have been employed previously in attempts to quantify the AOB. These methods include most-probable-number, competitive enzyme-linked immunosorbant monoclonal antibody assay, in situ hybridization and competitive PCR and have been described by Hermansson *et al.* (2001). Hermansson *et al.* (2001) also describe a real-time PCR for detection and quantification of AOB. This method has been employed in the current study to circumvent some of the difficulties of trying to quantify AOB in extremely diverse communities.

Molecular techniques have been used to investigate AOB populations from a variety of soil and sediment environments, including those around fish-farms (McCaig, Phillips, Stephen, Kowalchuk, Harvey, Herbert, Embley, Prosser, 1999; Stephen, McCaig, Smith, Prosser, Embley, 1996), but these studies have not assessed temporal changes in AOB communities. The assessment of the effects of farms over entire farm and fallowing cycles is necessary to demonstrate the sustainability or otherwise of farming practices. This study is also unique in that AOB communities have not been previously assessed in Tasmanian salmon farm sediments. Aquaculture sediments in Tasmania have not shown chemical responses (redox, sulphides, etc.) as extreme as those exhibited by sediments in the northern hemisphere (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004).

The aim of this study was to assess the effects of highly labile organic loading from salmon farms on the sediment AOB community. To achieve this end 16S rDNA

techniques have been employed to monitor shifts in AOB diversity and AOB numbers and to identify some members of the sediment AOB community over two 12-month farm cycles.

5.2 Methods

5.2.1 Sampling design

Samples were collected from two salmon farms as described in chapter 1.4.4.

5.2.2 DNA extraction and purification

DNA extraction from sediments was completed as described in chapter 3.2.2.

5.2.3 Polymerase Chain Reaction (PCR)

To avoid band doublets caused by redundant primers (McCaig, Phillips, Stephen, Kowalchuk, Harvey, Herbert, Embley, Prosser, 1999) a nested PCR approach was used to amplify 16S rDNA fragments from AOB. First 16S rDNA gene fragments from β -proteobacteria AOB were amplified using a 1650 Air Thermo-Cycler (Idaho Technology, Idaho Falls, Idaho) with the primers CTO 189fA/B (GAGRAAAGCAGGGGATCG) and CTO654r (CTAGCYTTGTAGTTTCAAACGC) (Kowalchuk, Stephen, De Boer, Prosser, Embley, Woldendorp, 1997). PCR products obtained using AOB specific primers were then amplified using universal primers 357f with a GC clamp (CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCTACGGGAG GCAGCAG) and 518r (GTATTACCGCGGCTGGTG) (Muyzer, de Waal, Uitterlinden, 1993). Reactions were performed using Clontech's Advantage 2 Polymerase Kit in accordance with the manufacturer's recommendations: Reactions of 50 μ l contained 10 x Advantage Taq PCR 2 Reaction Buffer (Clontech), 1 μ l of Advantage PCR 2 Taq DNA polymerase (Clontech.), 1 μ l of template DNA (0.1 – 0.5 ng/ μ l), 10 pmol of each primer, 1.25mM of each deoxyribonucleoside triphosphate. Thermal cycling for the amplification of products using the CTO primer set was carried out with an initial denaturation step of 95°C for 4 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min; cycling was completed by a final elongation step of 72°C for 4 min.

Thermal cycling for amplification using the universal primer set was carried out with an initial denaturation step of 95°C for 4 min followed by 20 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min; cycling was completed by a final elongation step of 72°C for 4 min. The presence and size of the amplification products were checked by agarose (1%) gel electrophoresis of the reaction product and ethidium bromide staining.

5.2.4 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed using the D-code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, USA) according to the method for perpendicular gels in the Bio-Rad manual. Samples were initially analysed with a 20% - 80% denaturing gradient using an 8% acrylamide gel. Samples were then re-run using a gradient of 40 – 70% for better band separation. Approximately 25µl of PCR product (with 5µl of 5X DGGE gel loading buffer) was loaded and the gel run at 60°C for 16 h. Gels were cooled then stained for 20 min. using 10µl of 10000x Sybr-gold nucleic acid stain (Molecular Probes, Eugene, USA) in 100ml Tris-acetate EDTA (TAE) buffer (40mM Tris-acetate; 1mM disodium EDTA; pH 8). Gels were imaged using a UV transilluminator and digital images captured on a Kodak DC60 digital camera fitted with a deep yellow #15 filter (Tiffen, Hauppauge, NY, USA). Bands were excised using a sterile scalpel blade, placed in a microcentrifuge tube and washed with 200ul of sterile MilliQ for 30 min to avoid external DNA contamination. DNA was then eluted from excised bands by soaking in 200ul of STE buffer overnight at 37°C.

5.2.5 Analysis of DGGE fingerprints

DGGE fingerprints were analysed as explained in chapter 4.2.4.

5.2.6 PCR re-amplification of DGGE band DNA

Duplicate DGGE bands were extracted from the same vertical positions but in different lanes from the DGGE gels. This was done to ensure that bands at the same position on the gels could be considered to be the same phylotype. One µl of band eluent was re-amplified using primers 357f and 518r in the Hotstart PCR Kit (Quiagen, Hilden, Germany). PCR was performed with a 15 minute, 95°C “hotstart”

step, followed by 25 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, with a final 4 minute 72°C step. Reamplified DNA product was then purified using the Prep-A-Gene[®] DNA purification system prior to sequencing.

5.2.7 Sequencing and Phylogenetic analysis

Direct sequencing of PCR products amplified from DGGE bands was carried out using the CEQ Dye Terminator Cycle Sequencing (DTCS) with Quick Start Kit (Beckman-Coulter, Berkely, USA). Reactions were carried out using a modification of the manufacturer's protocol; reactions were performed in 10 µl volumes using 2 µl of DTCS Quick Start Master Mix, 1 µl of primer (1.6 pmol/µl), and reactions were resuspended in 30 µl of Sample Loading Solution after ethanol precipitation.

Sequencing reactions were analysed using a Beckman CEQ2000 automated DNA sequencer, and electrophoretograms were manually checked and sequence data imported into a database using the BIOEDIT program (Hall, 1999). Sequences were compared to sequences in the GenBank database (<http://www.ncbi.nlm.nih.gov/blast>) using The Basic Local Area Search Tool (BLASTn) (Altschul, Madden, Schäffer, Zhang, Zhang, Miller, Lipman, 1997)). Sequences from this study were then aligned to reference sequences obtained from GenBank, using the Bioedit program and ClustalW (Hall, 1999; Thompson, Higgins, Gibson, 1994).

The Maximum Likelihood algorithm in the DNADIST program was used to generate a similarity matrix which was then applied to the NEIGHBOR program to generate similarity trees using the Neighbor Joining method. Both programs are included in the PHYLIP (Felsenstein, 1993) suite of programs.

5.2.8 Real-time PCR

Real time PCR was performed using primers specific for AOB on farm sediment samples (see 4.2.2). Standard curves were generated using an AOB sequence PCR fragment eluted and re-amplified from a pilot gel. It was decided to use a known fragment to generate standard curves to ensure samples and standards had the same reaction efficiency. DNA concentrations obtained from standard curves were converted to fragment copy number and ultimately AOB cell number assuming that all AOB have only one *rrn* operon (Hermansson, Lindgren, 2001). This has been

shown for all AOB cultured so far (Aakra, Utaker, Nes, 1999; Hermansson, Lindgren, 2001).

PCR reactions were performed in 20 µl volumes using single 100 µl strip tubes (Corbett Research, Australia). PCR reactions were performed using the Rotor-Gene thermocycler (Corbett Research, Australia), and data analysed using the Rotorgene software (V. 5.0). Reactions were run in 20 µl volumes containing 2 µl of 10 x Advantage Taq PCR 2 Reaction Buffer (Clontech), 0.4 µl of Advantage PCR 2 *Taq* DNA polymerase (Clontech.), 0.4 µl of template DNA (0.1 – 0.5 ng/µl), 5 pmol of each primer (either CTO189fA/Bf and CTO654r or 519f (CAG CMG CCG CGG TAA TAC) and 907r, 1.25mM of each deoxynucleoside triphosphate and SYBR green nucleic acid stain (Molecular probes) at a final concentration of 1:40,000. Assays were performed using a four step thermocycling program consisting of an initial 5 minute 95°C incubation followed by 35 cycles of a denaturation step of 30 seconds at 95°C, annealing of primers for 30 seconds at 55°C, elongation for 30 seconds at 72°C with fluorescent acquisition, and a further fluorescent acquisition step at 80°C. The temperature at which fluorescence analysis was performed was determined by examination of the melting profile of a number of samples, and performed at a temperature at which all primer dimer had melted, but the specific product had not (80°C). Standards for calibration of the real-time PCR assay were added to each assay, and included a dilution series of positive controls. All real-time PCR products were examined using agarose gel electrophoresis to ensure products corresponded to the correct size, and to ensure the absence of non-specific product. Samples were run in duplicate and analysis repeated if variance exceeded 10%.

5.2.8.1 Univariate statistical analysis

Analysis of variance (ANOVA) was used to test for the effect of farm (2 levels) and time (3) on AOB numbers. Data was analysed as explained in chapter 1.4.4 (Basic sampling design). Homogeneity of variances was checked visually by examining residual plots. Data that did not meet this assumption of ANOVA were log transformed. Significant factors were then compared using Tukeys HSD. All statistical tests were tested at $\alpha = 0.05$. The statistical software SPSS v10 was used to perform tests.

5.3 Results

5.3.1 AOB diversity at Farm 1

Generally, the DNA extraction, amplification and DGGE analysis was successful at farm one (Figure 5-1). Bands were generated in all samples. Real-time PCR quantification of AOB in sediments was also successful. Samples were again considered separately for each farm cycle because of inconsistent farm management practices over the period of the trial, as explained above in chapter 1.4.3.

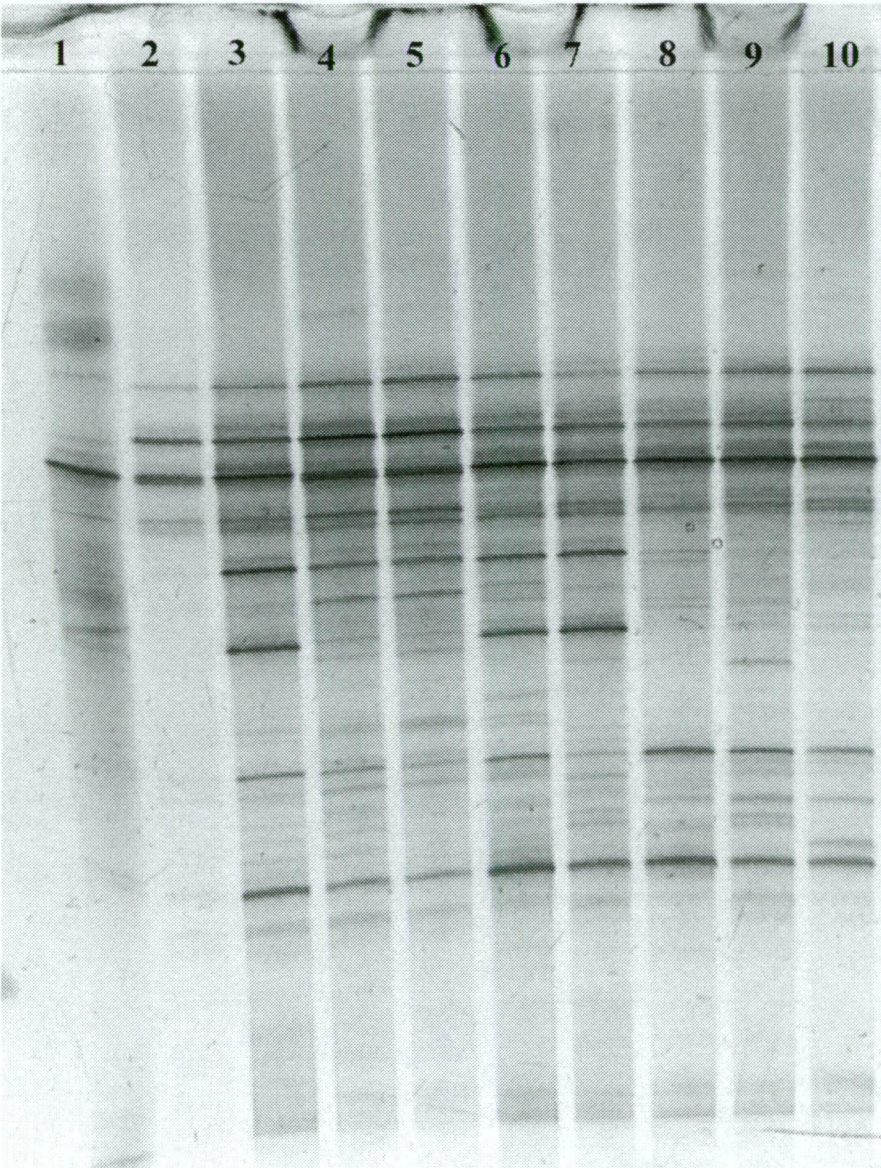


Figure 5-1 Inverted representative DGGE gel using AOB primers CTOF-CTOR and 357F – 518R. Lane 1: PCR positive control. Lane 2: Farm 1, Reference 1, 12 months. Lane 3: Farm2, Cage 1, T12. Lane 4: Farm2, Reference 2, T12. Lane 5: Farm 2, Reference 2, T12. Lane 6:

Farm 2, Cage 2, T12. Lane 7: Farm2, Cage 1, T24. Lane 8: farm 2, Cage 2, T24. Lane 9: Farm 2, Cage 2A, T0. Lane 10: Farm 2, Cage 2A, T0.

5.3.1.1 Stocking cycle 1

For the first 12 month stocking cycle the MDS plot is shown in Figure 5- 2A. A significant interaction between the effects of treatment and time on AOB community structure was observed ($F_{2,26} = 5.6$, $p=0.001$). The subsequent 1-way ANOSIM results for each pair of treatment/time combinations are given in Table 5- 1. Cage site AOB communities are not significantly different from those observed at reference sites at the beginning of the trial or after the nine month stocking period. After the 3 month fallowing period, however, AOB communities are different at cage and reference sites. Paired comparisons of reference site communities demonstrate that there is no significant difference between the communities at the start of the trial and after nine months, or between the sites at nine months and at the end of the 12 month cycle. There is, however, a difference between the reference site communities at the beginning and end of the 12 month cycle. Paired comparisons of cage site communities show that they were all significantly different at each sampling time.

Table 5-1 ANOSIM results for farm 1, cycle 1 AOB community fingerprints. T2 = 2 months; T9 = 9 months T12 = 12months.

Groups	R Statistic	Significance Level (%)
Ref T2, Cage T2	0.313	5.6
Ref T9, Cage T9	0.28	8.7
Ref T12, Cage T12	0.616	0.4
Ref T2, Ref T9	0.093	17.7
Ref T9, Ref T12	0.272	6.3
Ref T2, Ref T12	0.94	0.2
Cage T2, Cage T9	1.0	0.8
Cage T9, Cage T12	1.0	0.2
Cage T2, Cage T12	1.0	0.5

5.3.1.2 Farm 1 Stocking cycle 2

5.3.1.2.1 Cage1/reference 1

During the second farm cycle there was no significant interaction between treatment and time and no effect of treatment on AOB communities. There was, however a

significant effect of time ($F_{2,11} = 13.2, p=0.0001$) on AOB community composition at both cage and reference sites (Figure 5- 2B). Pairwise tests for the effect of time showed that the AOB communities differed from one another at all times during this 12 month farming cycle.

5.3.1.2.2 Cage2/reference 2

Similar results were observed for cage 2 and reference 2 samples during the second 12 month cycle. No significant interaction was detected and treatment did not have a significant effect on AOB community structure. Sampling time, though, did significantly affect AOB community composition ($F_{2,12} = 15.5, p = 0.0001$) (Figure 5- 2C). Again pairwise tests of the different levels of sampling time showed that all were significantly different from one another.

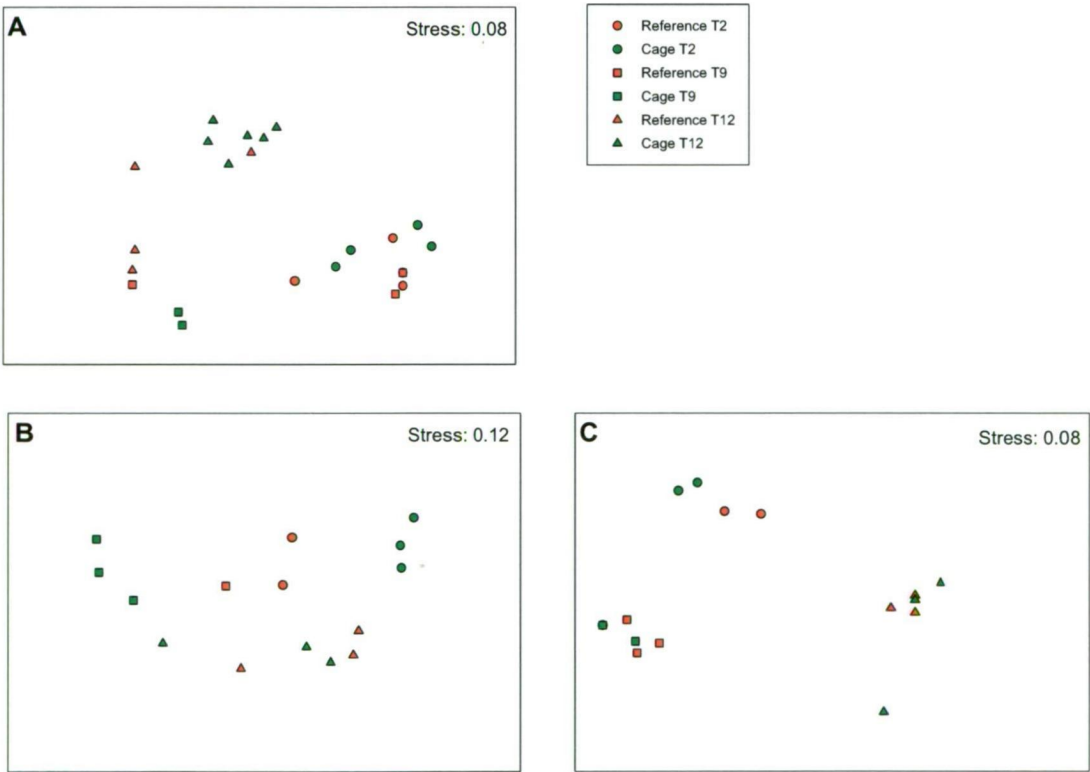


Figure 5-2 MDS plots showing AOB community shifts at farm 1 during the 24 month study. A) Cage and reference sites during stocking cycle 1 (insert dates), B) Cage 1/Reference 1, stocking cycle 2 (insert dates), and C) Cage 2/Reference 2, stocking cycle 2 (insert dates). For A, T2 = 2 months, T9 = 9 months T12 = 12months, for B T2 = 12 months, T9 = 21 months T12 = 24months, for C T2 =13.5 months, T9 = 21.5 months T12 = 25.5 months. N = 3.

5.3.2 AOB numbers at Farm 1

During the first stocking cycle both treatment ($F_{25,1} = 13.67$, $p = 0.001$) and time ($F_{2,25} = 5.15$, $p = 0.013$) had a significant effect on sediment AOB numbers. AOB numbers were lower at cage sites and declined at both sites over the 12 month period (Table 5-2). During the second 12 month cycle at cage 1 and reference 1 however, treatment had no effect on AOB population density, but time did ($F_{2,12} = 7.62$, $p = 0.007$). Again AOB numbers declined over the trial period. At cage 2 and reference 2 there was significant interaction effect ($F_{2,15} = 5.22$, $p = 0.031$) of treatment and time on AOB numbers. AOB numbers appeared to remain steady at the reference site for the initial 9 months, but declined during the final 3 months. At cage 2 the numbers increased during the 12 month cycle.

Table 5-2 Mean AOB numbers/g sediment (\pm se) at Farm 1 over 2 production cycles. T2 = first sampling period of cycle, T9 = end of stocking period, T12 = end of fallow period. N = 3.

	Farm Cycle 1		Farm Cycle 2			
Time	Cage	Reference	Cage 1	Reference 2	Cage 2	Reference 2
T2	$2.0 \times 10^7 \pm$	$4.3 \times 10^7 \pm$	$1.2 \times 10^7 \pm$	$1.8 \times 10^7 \pm$	$8.9 \times 10^6 \pm$	$1.8 \times 10^7 \pm$
	5.3×10^6	9.5×10^6	1.3×10^6	5.5×10^6	1.6×10^6	3.1×10^6
T9	$1.9 \times 10^7 \pm$	$3.3 \times 10^7 \pm$	$1.5 \times 10^7 \pm$	$1.2 \times 10^7 \pm$	$1.2 \times 10^7 \pm$	$1.8 \times 10^7 \pm$
	3.8×10^6	3.7×10^6	7.8×10^5	2.5×10^5	1.0×10^6	4.0×10^6
T12	$1.5 \times 10^7 \pm$	$1.9 \times 10^7 \pm$	$7.3 \times 10^6 \pm$	$9.2 \times 10^6 \pm$	$1.5 \times 10^7 \pm$	$9.3 \times 10^6 \pm$
	2.4×10^6	2.8×10^6	1.0×10^6	7.8×10^5	2.2×10^6	1.6×10^6

5.3.3 AOB diversity at Farm 2

During the first 12 month farm cycle at farm 2 both treatment ($R = 0.613$, $p = 0.001$) and cages ($R = 0.424$, $p = 0.001$) had a significant effect on sediment AOB community. No interaction effect was detected. Figure 5- 3A shows an nMDS plot of the AOB community data demonstrating that reference and cage site communities differed from one another and that AOB communities at the beginning of the trial and after nine months of stocking did not differ significantly, but after three months following the AOB community had shifted significantly (Table 5- 3).

Table 5-3 ANOSIM results for the effect of time on AOB community at farm 2, cycle 1. T2 = 0 months, T9 = 9 months T12 = 12months.

Group	R statistic	Significance (%)
T2, T9	0.087	20.5
T2, T12	0.726	0.1
T9, T12	0.406	0.1

Over stocking cycle two there was a significant interaction between the effects of treatment and time on sediment AOB community ($F_{2,30} = 7.5$, $p = 0.001$) (Figure 5-3B). The results of ANOSIM comparisons of treatment/time combinations are shown in Table 5- 3.

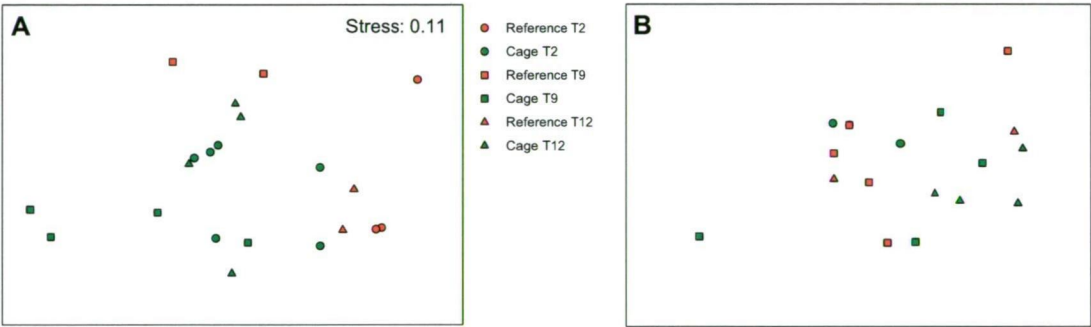


Figure 5-3 A) nMDS plot showing shifts in AOB community at farm 2 over stocking cycle 1 (insert dates). T2 = two months, T9 = 9 months, T12 = 12 months. B) CAP plot of first two canonical axes of farm two, cycle two. T2 =12 months, T9 = 21 months T12 = 24 months.

Cage site AOB communities differed from those at reference sites at the beginning of the trial, moved further away during the nine months of stocked cages and approached the community at reference sites after the fallowing period. At Reference sites the community showed a shift over the 9 month stocking period and a counter shift during fallowing. At cage sites, though, the AOB community showed greater variation and thus appeared to exhibit smaller shifts (lower R statistic) over the farming cycle.

Table 5-4 ANOSIM post hoc comparisons for farm 2, cycle 2. T2 = 0 months, T9 = 9 months T12 = 12months.

Groups	R statistic	Significance (%)
Ref T0, Cage T0	0.400	2.6
Ref T9, Cage T9	0.700	0.2
Ref T12, Cage T12	0.256	7.4
Ref T0, Ref T9	0.700	0.2
Ref T9, ref T12	0.500	49.2
Ref T0, Ref T12	0.625	0.2
Cage T0, Cage T9	0.116	15.2
Cage T9, Cage T12	0.168	9.5
Cage T0, Cage T12	0.257	5.0

5.3.4 AOB numbers at Farm 2

During the first 12 month cycle at farm 2 AOB numbers were affected by time ($F_{2,28} = 10.994$, $p = 0.000$), but not by organic loading from the farm. AOB numbers declined over the nine month stocking period and remained steady during the 3 month fallowing period (Table 5- 5). During the second farm cycle both treatment and time had no significant effect on AOB numbers. AOB numbers remained at a similar level to that seen at the end of the first cycle at both farm and reference sites (Table 5- 5).

Table 5-5 Mean AOB numbers/g sediment (± se) at Farm 2 over 2 production cycles. T2 = first sampling period of cycle, T9 = end of stocking period, T12 = end of fallow period. N = 3.

	Farm Cycle 1		Farm Cycle 2	
Time	Cage	Reference	Cage	Reference
T2	2.8 x 10 ⁸ ±	1.0 x 10 ⁸ ±	2.4 x 10 ⁷ ±	2.4 x 10 ⁷ ±
	6.1 x 10 ⁷	1.5 x 10 ⁷	1.0 x 10 ⁷	9.7 x 10 ⁶
T9	2.0 x 10 ⁷ ±	3.2 x 10 ⁷ ±	3.0 x 10 ⁷ ±	5.8 x 10 ⁷ ±
	4.6 x 10 ⁶	6.0 x 10 ⁶	5.6 x 10 ⁶	1.8 x 10 ⁷
T12	3.1 x 10 ⁷ ±	2.4 x 10 ⁷ ±	1.9 x 10 ⁷ ±	2.3 x 10 ⁷ ±
	3.2 x 10 ⁶	9.7 x 10 ⁶	6.8 x 10 ⁶	1.3 x 10 ⁷

5.3.5 DGGE band sequencing/AOB identities

In order to avoid the band doublets caused by degenerate primers experienced in other studies of AOB using DGGE (McCaig, Phillips, Stephen, Kowalchuk, Harvey, Herbert, Embley, Prosser, 1999) a nested PCR approach was employed. Representative bands generated from DGGE gels were excised and sequenced successfully. All sequences/bands clustered with known marine, sediment and soil β-subgroup proteobacterial sequences (Figure 5- 4). (McCaig, Phillips, Stephen, Kowalchuk, Harvey, Herbert, Embley, Prosser, 1999; Stephen, McCaig, Smith, Prosser, Embley, 1996) reported the presence of sequences from β-proteobacterial AOB cluster 5 and attributed these sequences to organically enriched environments. No observed sequences from this study grouped strongly with these sequences (Sequences EnvA-21, EnvA2-13 and AF006666 in Figure 5-4).

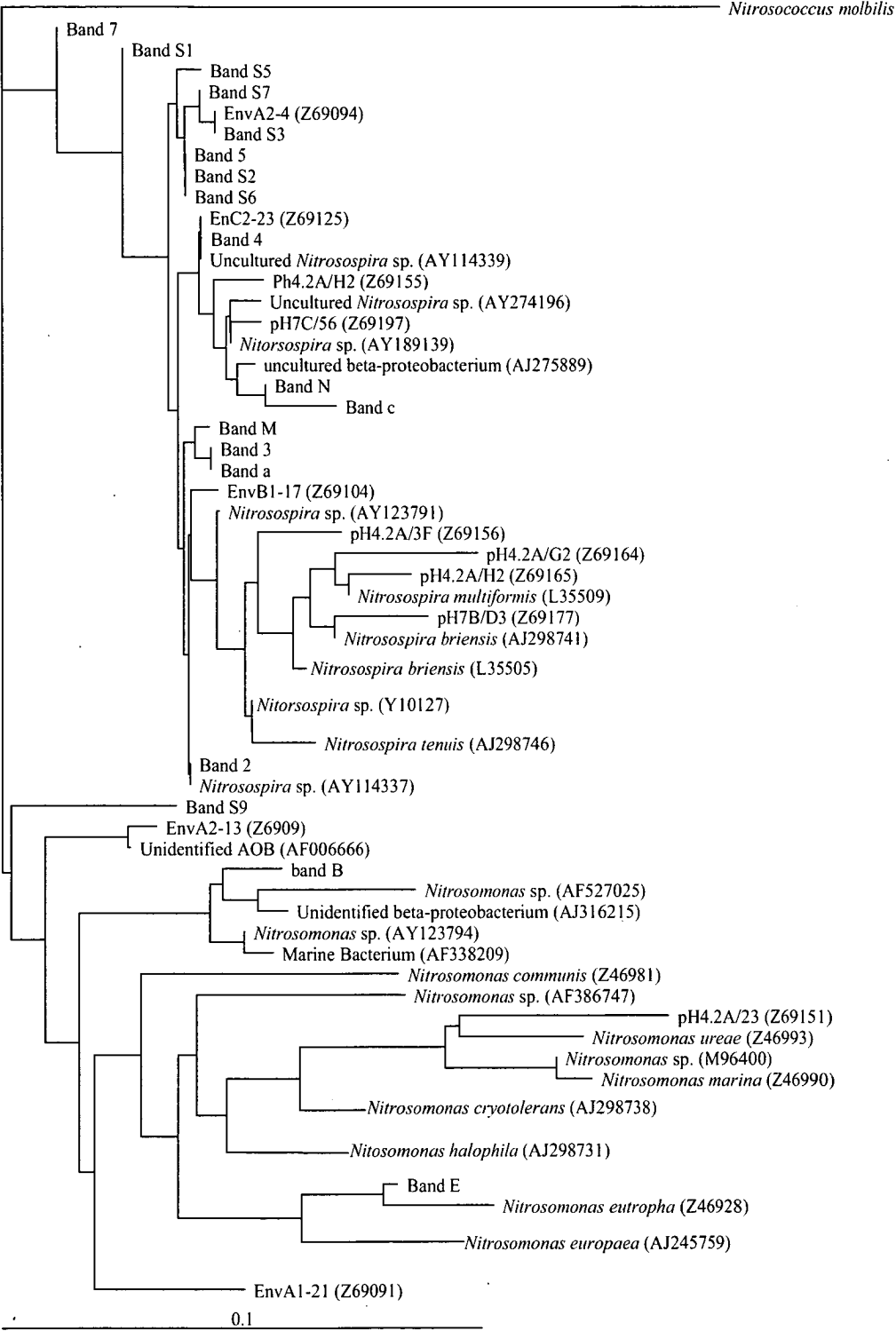


Figure 5-4 Neighbour-joining similarity tree showing the relationship of excised DGGE bands to reference beta-proteobacteria ammonia-oxidisers.

5.4 Discussion

This study investigated shifts in the AOB communities at two salmon farms during commercial farming operations. Farming conditions included two cycles of nine months intensive farming followed by three months fallowing. The aim of the study was to assess farm induced shifts in the AOB community and to assess the efficacy of farm management practices, such as short term cage rotation/fallowing, in maintaining a diverse AOB community and thus ensuring at least the potential for nitrification. This study targeted only members of the β -subgroup AOB. Molecular analysis did indicate shifts in the AOB community and AOB numbers over the duration of the trial at both sites. At farm 1 there appeared to be only a weak effect of the farm on the AOB community, but a strong effect of time. It should be noted that although there is a significant interaction between treatment and time and a significant difference between the cage and reference communities at the end of the first 12 month period, the mid-value R statistic (Table 5- 1) suggests that the communities are not completely differentiated. During the second farm cycle no such interaction occurred and the effect of the farm is lost.

At Farm 2 there was an effect of both the farm and time on AOB communities. Over the first cycle the AOB communities at the cage sites differed from those at the reference sites and both AOB communities differed through time. The major shift occurred during the 3 month fallow period. During the second cycle the communities approached each other at the end of the trial, after shifting further apart during the stocking period.

It is evident that interpretation of multivariate data concerning microbial diversity is difficult, especially if there is a temporal component to deal with. Employing several multivariate techniques (nMDS, CAP and cluster analysis) has aided in the interpretation of the above data. In terms of AOB diversity, as assessed with DGGE banding pattern analysis, it is evident that the sediments investigated in this study are very dynamic. Most previous studies investigating AOB in natural communities have contained no temporal aspect, that is they used only one sampling time (for example (Freitag, Prosser, 2003; Kowalchuk, Stephen, De Boer, Prosser, Embley,

Woldendorp, 1997; Kowalchuk, Sienstra, Heilig, Stephen, Woldendorp, 2000; Phillips, Smith, Embley, Prosser, 1999; Rowan, Snape, Fearnside, Barer, Curtis, Head, 2003; Stephen, McCaig, Smith, Prosser, Embley, 1996; Stephen, Kowalchuk, Bruns, McCaig, Phillips, Embley, Prosser, 1998)). de Bie *et al.* (2001) however demonstrated that AOB populations were not necessarily stable over time. They correlated changes in AOB population structure to changes in chemical gradients. The AOB communities in the sediments studied here appear to be very dynamic and appear to shift, at both cage and reference sites, through time. Such community shifts are likely to be in response to changing environmental and sediment geochemical gradient conditions. What is evident at all sites and at all times is that a large amount of variation in AOB communities exists. This variation suggests that a wide variety of AOB phylotypes are present and are able to take advantage of changing conditions to dominate AOB communities at different times. Indeed nitrifiers are able to withstand long periods of dormancy (Kowalchuk, Bodelier, Heilig, Stephen, Laanbroek, 1998) allowing different phylotypes to await favourable conditions. Previous studies investigating changing microbial communities have reported similar events. Kaneko *et al.* 1977 (as referenced in (Atlas, Bartha, 1998)) found that although the species re-introduced into arctic seas after winter ice melts may have been different to species present when the ice formed, the niches filled by these bacteria remained constant. Although cage and reference sites often exhibited different AOB communities, both sites were comprised of diverse and dynamic AOB communities.

The nested-PCR approach greatly facilitated the interpretation of DGGE gel banding patterns by avoiding ambiguous bands, but resulted in shorter sequences (161bp) than those obtained in previous studies (McCaig, Phillips, Stephen, Kowalchuk, Harvey, Herbert, Embley, Prosser, 1999; Stephen, McCaig, Smith, Prosser, Embley, 1996). Although shorter sequences are ideal for DGGE analysis they result in less information and consequently less resolution for phylogenetic analysis. Although not enough resolution was obtained to identify the seven clusters of β -subgroup AOB reported elsewhere (McCaig, Phillips, Stephen, Kowalchuk, Harvey, Herbert, Embley, Prosser, 1999; Stephen, McCaig, Smith, Prosser, Embley, 1996) sequences from both cage and reference sites were diverse and clustered with sequences obtained from marine sediments and soil environments. No sequences clustered with

cluster 5 β -proteobacterial AOB sequences. Although sequences from this cluster have been associated with organically enriched fish-farm sediments (McCaig, Phillips, Stephen, Kowalchuk, Harvey, Herbert, Embley, Prosser, 1999; Stephen, McCaig, Smith, Prosser, Embley, 1996) they have also been found in nutrient poor environments (Kowalchuk, Stephen, De Boer, Prosser, Embley, Woldendorp, 1997).

AOB population densities at cage and reference sites also exhibited difficult-to-interpret variation over the trial. Farm operations appear to play a more important role in influencing AOB numbers at Farm1 than they do at farm 2. This is especially evident over the first stocking cycle, when AOB numbers are lower at the cage sites of farm 1. AOB numbers do, however, decline at both cage and reference sites over the first year. During the second cycle at cage 1/reference 1 there was no effect of farm, but an effect of time: AOB numbers declining throughout the cycle. At cage 2/reference 2, however, both farm operations and time interacted to affect AOB numbers: AOB numbers declined over the final 3 months of the trial at the reference site, but increased slightly at the farm site. At farm 2, no changes in AOB population numbers could be attributed to the farm. The sediment characteristics of both farms go some way to explaining the differing responses of the AOB communities to farm loading. The sediments at farm 1 are sandy and very low in carbon (0.2%) (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). The sediments at farm 2, on the other hand, are high in organic content (4%) and much finer in composition (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). The AOB communities at farm 1 are more likely to exhibit a large response to organic loading than those at farm 2, which are already conditioned to high nutrient conditions. Despite showing variations in response to farming activities the AOB populations at all sites are present in numbers that would be considered high using traditional enumeration techniques or normal in light of the real-time PCR method utilised here (Hermansson, Lindgren, 2001).

Although AOB numbers appeared relatively stable, their contribution to the total microbial population declined during farming. This apparent decline in the importance of the AOB community resulted from increases in total bacterial numbers, rather than a decline in AOB numbers. Given the slow growth rates of AOB and the fact that they are usually present only in relatively small numbers in the environment

(Hermansson, Lindgren, 2001) it is not surprising that AOB numbers did not increase markedly during the study period. Nitrification, and consequently possibly AOB numbers, are adversely affected by conditions often associated with fish-farm sediments. Studies of macrobenthic communities have demonstrated that benthic fauna associated with conditions of nutrient enrichment actually enhance benthic production via bioturbation (Heilskov, Holmer, 2001; Wu, Tsutsumi, Kita-tsukamoto, Kogure, Ohwada, Wada, 2003). Benthic fauna such as *Capitella* spp., which came to dominate the impacted sediments under cages at both farms in this study (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004), stimulate benthic microbial activity (Kristensen, 1988). Microbial activity is stimulated by increased sediment oxidation, increased substrate surface area and the presence of organic rich faecal pellets and secreted mucoid products (Heilskov, Holmer, 2001; Wu, Tsutsumi, Kita-tsukamoto, Kogure, Ohwada, Wada, 2003). Sediments in this study did not at any point become azoic (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). It is therefore likely that healthy AOB populations were maintained in the oxidised conditions created by the macrofauna. This idea is further supported by the fact that ammonia did not accumulate in the surface sediments inhabited by *Capitella* spp and sampled in this study (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). The effect of azoic conditions on AOB populations remains to be elucidated.

5.5 Conclusions

The results presented above indicate that fish-farm organic input is one of a number of factors that influence both AOB community composition and population density. AOB communities encountered were very diverse and present in relatively high numbers at both reference and farm sites at both farms. Previously it has been shown that organic loading from fish-farms results in the formation of anaerobic sediments that do not support any nitrification or denitrification even though AOB DNA has been amplified and identified from these sediments (McCaig, Phillips, Stephen, Kowalchuk, Harvey, Herbert, Embley, Prosser, 1999; Stephen, McCaig, Smith, Prosser, Embley, 1996). Unlike those studies the sediments investigated here did not accumulate ammonia in the uppermost sediment layers (0 – 0.5cm) (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004), suggesting that

although nitrification/denitrification were not measured they might have been occurring.

AOB communities from different sediment types also appeared to respond differently to fish-farm waste. The communities inhabiting carbon-poor sandy sediments showed the greatest response to organic loading. This result is consistent with that shown by the benthic macrofauna (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004) and suggests that the AOB community inhabiting the carbon rich silt-sediment is more adapted to conditions induced by farm inputs and therefore exhibited less change. It may also be said that the communities in the sandy sediment had to endure a greater change than those in the fine sediments and therefore elicit a more discernable response.

It would appear then, that the AOB communities in Tasmanian fish-farm sediments are resilient enough to cope with the changes in conditions caused by farming at levels experienced in this trial. The effects of more extreme eutrophication, as is experienced in many other salmon farming locations throughout the world, was not encountered in this study and so has not been investigated.

Chapter Six

***Cytophaga-Flavobacteria* diversity in two salmon farm sediments**

Chapter 6 *Cytophaga-Flavobacteria* diversity in two salmon farm sediments

6.1 Introduction

Sediments exhibit a broad array of microbial diversity, to the extent that almost every bacterial division is represented in sediments to some degree (Bowman, McCuaig, 2003; Hugenholtz, Goebel, Pace, 1998; Kirchman, 2002; Torsvik, Sorheim, Goksoyr, 1996). Although sediments exhibit great bacterial diversity not all groups represented are abundant and/or contribute significantly to biogeochemical cycles (Kirchman, 2002). Until recently one group that has often been overlooked in sediment communities is the *Cytophaga-Flavobacteria-Bacteroidetes* (CFB) phylum. It is well known that this group is strongly associated with the marine water column and marine aggregates, but it has only fairly recently been associated with sediment communities (Llobet-Brossa, Rossello-Mora, Amann, 1998; Ravensschlag, Sahm, Amann, 2001). The CFB group was also thought to be mainly important in aerobic environments, but it has been shown that the addition of complex organic substrates to anaerobic sediments resulted in the growth of members of CFB bacteria (Cytryn, Gelfand, Barak, van Rijn, Minz, 2003; Rossello Mora, Thamdrup, Schaefer, Weller, Amann, 1999). These studies concluded that the CFB group may be the primary catalysts of macromolecule hydrolysis and fermentation and thus play a pivotal role in the anaerobic, as well as aerobic, decomposition of complex organic matter.

The CFB are a diverse group that has been labelled inconsistently in the past (Kirchman, 2002). Other, commonly used labels include Bacteroidetes (*Ludwig, Klenk, 2001*) which will become the official nomenclature following the publication of the edition of new Bergey's Manual of Systematic Bacteriology. The CFB are characterised in general by a number of phenotypic and genotypic traits including a Gram negative type cell wall, general inability to form spores (exception genus *Sporocytophaga*), frequent ability to move by gliding, and generally rod-like to filamentous morphology. Many members of the CFB form pigments. The CFB are often adept at degrading high molecular weight biopolymers such as cellulose, chitin and pectin (Reichenbach, Dworkin, 1991). Early sequence analysis of 16S rRNA genes divided the CFB into two clusters (Woese, 1987) represented by the genera: *Bacteroides* and *Flavobacterium*. Even though the number of available sequences has risen markedly since Woese's review, the majority of marine CFB species still cluster

together (e.g. family Flavobacteriaceae) (Kirchman, 2002). The issue of phylogenetic characterisation is of great importance for molecular studies examining members of the CFB. In order to design primers/probes for a particular group of bacteria it is necessary for the bacteria of interest to group together, without any other bacteriotypes. The most commonly used oligonucleotide probe for investigating members of the CFB cluster is CF319a (Manz, Amann, Ludwig, Vancanneyt, Schleifer, 1996). CF319a binds to rRNA from the majority of the CFB (Kirchman, 2002). Kirchman(2002) has noted that probe CF319a does not effectively bind to a few members of the CFB and so may result in studies using this probe underestimating the contribution of the CFB. Weller (Weller, Glockner, Amann, 2000) and Abell (2005) successfully used a primer specific to many marine CFB. This primer, 558f, was utilized in this study to amplify 16S rDNA from CFB present in marine sediments. For the remainder of this chapter CFB refers to members of the CFB that are targeted by primer 558f

Sediments under salmon farms receive large amounts of organic matter from uneaten fish food, faecal excretions and fouling communities. This organic loading may result in increased microbial production, the development of oxygen depleted conditions and production of toxic metabolites. Generally these conditions are seen as deleterious to the sediment ecosystem. Several factors determine the extent to which these deleterious effects occur including; the organic load experienced, the duration of the load and the rate at which organic matter is turned over. Cytryn *et al.* (2003) and Rossello-Mora *et al.* (1999) reported that the CFB may be instrumental in maintaining benthic production by supplying hydrolysis and fermentation products to other bacterial groups. The CFB may then be important as the initial degraders of organic matter in fish-farm sediments and their presence may enhance the assimilative capacity of these sediments.

This study has utilised molecular techniques to investigate the diversity and relative abundance of the CFB in marine sediments under fish-farms. The CFB were analysed over two production cycles of nine months farming, three months following to investigate the role these bacteria may play in organically loaded sediments and their recovery following cessation of loading.

6.2 Methods

6.2.1 DNA extraction and purification

DNA was extracted from sediments as described in chapter 3.2.2.

6.2.2 Polymerase Chain Reaction (PCR) and

834 bp fragments of the 16S rRNA gene were enzymatically amplified using a 1650 Air Thermo-Cycler (Idaho Technology, Idaho Falls, Idaho) with the primers 558F (ATT GGG TTT AAA GGG TCC) (Weller, Glockner, Amann, 2000) and 1392RC (CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CAC GGG CGG TGT GTA C) (Ferris, Muyzer, Ward, 1996). A GC clamp was attached to the reverse primer in order to increase DGGE gel separation. Reactions were performed using Advantage 2 Polymerase Kit (Clontech) in accordance with the manufacturer's recommendations: Reactions of 50 µl contained 10 x Advantage Taq PCR 2 Reaction Buffer, 1 µl of Advantage PCR 2 *Taq* DNA polymerase, 1 µl of template DNA (0.1 – 0.5 ng/µl), 10 pmol of each primer, 1.25mM of each deoxynucleoside triphosphate. Thermal cycling was carried out with an initial denaturation step of 95°C for 4 min followed by 19 cycles of denaturation at 95°C for 1 min, annealing at 64°C for 1 min (decreasing by –0.5°C every cycle), and elongation at 72°C for 2 min and 10 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and elongation at 72°C for 2 min; cycling was completed by a final elongation step of 72°C for 4 min. Amplification products were checked visually by agarose (1%) gel electrophoresis.

6.2.3 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed using the D-code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, USA) according to the method for perpendicular gels in the Bio-Rad manual. Samples were initially analysed with a 20% - 80% denaturing gradient using an 8% acrylamide gel. Samples were then re-run using a gradient of 20 – 50% for better band separation. Approximately 25µl of PCR product (with 5µl of 5X DGGE gel loading buffer) was loaded and the gel run at 80V, 60°C for 16 h. Gels were cooled then stained for 20 min. using 10µl of 10000x Sybr-gold nucleic acid

stain (Molecular Probes, Eugene, USA) in 100ml Tris-acetate EDTA (TAE) buffer (40mM Tris-acetate; 1mM disodium EDTA; pH 8). Gels were imaged using a UV transilluminator and digital images captured on a Kodak DC60 digital camera fitted with a deep yellow #15 filter (Tiffen, Hauppauge, NY, USA). Bands were excised using a sterile scalpel blade, placed in a microcentrifuge tube and washed with 200ul of sterile MilliQ water for 30 min to avoid external DNA contamination. DNA was then eluted from excised bands by soaking in 200ul of STE buffer overnight at 37°C.

6.2.4 Analysis of DGGE fingerprints

DGGE fingerprint analysis was undertaken as described in chapter 4.2.4.

6.2.5 PCR re-amplification of DGGE band DNA

Duplicate DGGE bands were extracted from the same vertical positions but in different lanes from the DGGE gels. This was done to ensure that bands at the same position on the gels could be considered to be the same phylotype. One µl of band eluent was re-amplified using primers 558f and 1392r in the Hotstart PCR Kit (Quiagen, Hilden, Germany). PCR was performed with a 15 minute, 95°C “hotstart” step, followed by 25 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, with a final 4 minute 72°C step. Reamplified DNA product was then purified using the Prep-A-Gene[®] DNA purification system prior to sequencing.

6.2.6 Sequencing and Phylogenetic analysis

Direct sequencing of PCR products amplified from DGGE bands was carried out using the CEQ Dye Terminator Cycle Sequencing (DTCS) with Quick Start Kit (Beckman-Coulter, Berkely, USA). Reactions were carried out using a modification of the manufacturer’s protocol; reactions were performed in 10 µl volumes using 2 µl of DTCS Quick Start Master Mix, 1 µl of primer (1.6 pmol/µl), and reactions were resuspended in 30 µl of Sample Loading Solution after ethanol precipitation. Sequencing reactions were analysed using a Beckman CEQ2000 automated DNA sequencer, and electrophoretograms were manually checked and sequence data imported into a database using the BIOEDIT program (Hall, 1999). Sequences were compared to sequences in the GenBank database (<http://www.ncbi.nlm.nih.gov/blast>)

using The Basic Local Area Search Tool (BLASTn) (Altschul, Madden, Schäffer, Zhang, Zhang, Miller, Lipman, 1997)). Sequences from this study were then aligned to reference sequences obtained from GenBank, using the Bioedit program and ClustalW (Thompson, Higgins, Gibson, 1994).

The Maximum Likelihood algorithm in the DNADIST program was used to generate a similarity matrix which was then applied to the NEIGHBOR program to generate similarity trees using the Neighbor Joining method. Both programs are included in the PHYLIP (Felsenstein, 1993) suite of programs.

6.2.7 Real-time PCR

Real time PCR was performed on sediment samples using primers specific for CFB (see 6.2.2). Standard curves were generated using an CFB sequence PCR fragment eluted and re-amplified from a pilot gel. It was decided to use a known fragment to generate standard curves to ensure samples and standards had the same reaction efficiency. DNA concentrations obtained from standard curves were not converted to fragment copy number, and ultimately CFB cell number, because it has not been shown that all CFB have only one *rrn* operon. Instead amounts of CFB DNA are used to infer changes in population number.

PCR reactions were performed in 20 µl volumes using single 100 µl strip tubes (Corbett Research, Australia). PCR reactions were performed using the Rotor-Gene thermocycler (Corbett Research, Australia), and data analysed using the Rotorgene software (V. 5.0). Reactions were run in 20 µl volumes containing 2 µl of 10 x Advantage Taq PCR 2 Reaction Buffer (Clontech), 0.4 µl of Advantage PCR 2 *Taq* DNA polymerase (Clontech.), 0.4 µl of template DNA (0.1 – 0.5 ng/µl), 5 pmol of each primer (558f and 907r), 1.25mM of each deoxynucleoside triphosphate and SYBR green nucleic acid stain (Molecular probes) at a final concentration of 1:40,000. Assays were performed using a four step thermocycling program consisting of an initial 5 minute 95°C incubation followed by 35 cycles of a denaturation step of 30 seconds at 95°C, annealing of primers for 30 seconds at 55°C, elongation for 30 seconds at 72°C with fluorescent acquisition, and a further fluorescent acquisition step at 80°C. The temperature at which fluorescence analysis was performed was

determined by examination of the melting profile of a number of samples, and performed at a temperature at which all primer dimer had melted, but the specific product had not (80°C). All real-time PCR products were examined using agarose gel electrophoresis to ensure products corresponded to the correct size, and to ensure the absence of non-specific product. Samples were run in duplicate and analysis repeated if variance exceeded 10%.

6.2.7.1 Univariate statistical analysis

Analysis of variance (ANOVA) was used to test for the effect of farm (2 levels) and time (3 levels) on CFB DNA concentrations. Data were analysed as explained in chapter 1.4.4 (basic sampling design). Homogeneity of variances was checked visually by examining residual plots. Data that did not meet this assumption of ANOVA were log transformed. Significant factors were then compared using Tukeys HSD. All statistical tests were tested at $\alpha = 0.05$. The statistical software SPSS v10 was used to perform these tests.

6.3 Results

6.3.1 CFB diversity at farm 1

Generally the DNA extraction, amplification and DGGE analysis was successful at farm 1 (Figure 6-1). Bands were generated in all samples. Samples are again considered separately for each cycle because of inconsistent farm management practices over the period of the trial, as explained above in chapter 1.4.3.

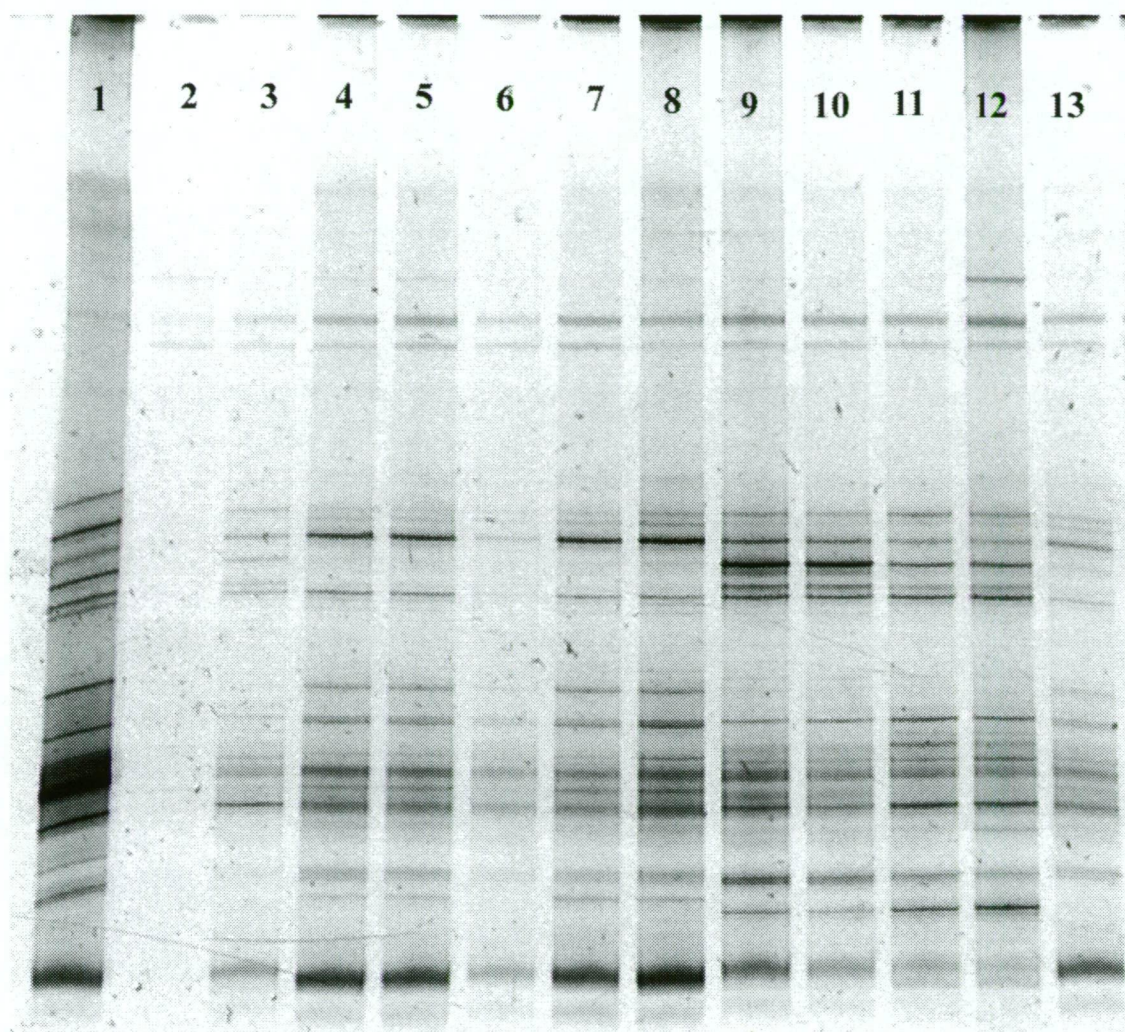


Figure 6-1 Inverted representative DGGE gel using CFB primers 519F – 907RC. Lane 1: PCR positive control. Lane 2: Farm 1, Reference 1, T0. Lane 3: Farm 1, Cage 1, T0. Lane 4: Farm 1, Reference 1, T2. Lane 5: Farm 1, Reference 1, T2. Lane 6: Farm 1, Reference 1, T2. Lane 7: Farm 1, Reference 2, T2. Lane 8: Farm 1, Reference 2, T2. Lane 9: Farm 1, Cage 1, T2. Lane 10: Farm 1, Cage 1, T2. Lane 11: Farm 1, Cage 2, T2. Lane 12: Farm 1, Cage 2, T2. Lane 13: Farm 1, Reference 1, T9.

6.3.1.1 Stocking cycle 1

For the first 12 month stocking cycle the nMDS plot is shown in Figure 6- 1A. A significant interaction was observed ($F_{2,27} = 15.8$, $p=0.001$) between treatment and time on CFB community dynamics. The subsequent 1-way ANOSIM results for pairs of planned comparisons of treatment/time combinations are given in Table 6- 1.

Reference site CFB communities were always different from those at cage sites. At reference sites the community showed a gradual shift up to the end of the stocking period, then a major shift during the fallowing period. At cage sites a shift occurs over the nine month stocking period. During the fallowing period no further shift occurs, but variation between replicates decreases markedly.

Table 6-1 ANOSIM values comparing the similarity between pairs of locations at farm 1 over stocking cycle 1

Groups	R Statistic	Significance Level (%)
Ref T2, Cage T2	0.844	0.8
Ref T9, Cage T9	0.928	0.2
Ref T12, Cage T12	1	0.2
Ref T2, Ref T9	0.403	3.5
Ref T9, Ref T12	1	0.2
Ref T2, Ref T12	0.936	0.2
Cage T2, Cage T9	1	0.5
Cage T9, Cage T12	0.567	0.2
Cage T2, Cage T12	0.905	0.5

6.3.1.1 Stocking cycle 2

For the second 12 month stocking cycle cage 1/reference 1 and cage 2/reference 2 combinations were considered separately.

6.3.1.1.1 Cage1 / Reference 1

The factors treatment and time showed a significant interaction ($F_{2,12} = 950.31$, $p=0.001$) in their effect on CFB community dynamics. ANOSIM results for comparisons of pairs of treatment/time combinations showed p values at the lower limit ($p = 10\%$), given the small number of permutations possible for the test. Given the high R-value for these tests it is highly likely that the groups are different, despite not achieving a p value less than 0.05. Figure 6- 2 shows a cluster diagram that aids in the interpretation of the nMDS plot (Figure 6- 1B). These groupings are somewhat more useful than the significance test in this instance, and demonstrate that at the highest level (55% similarity) the reference site at the beginning of the stocking period is different from all other groups. At around 70% similarity though, three groups appear to form, comprising 1) the reference sites at the beginning of the stocking period, 2) the cages at the beginning of the stocking period and the remaining reference sites and 3) the cages at the end of the stocking period and at the end of the trial.

6.3.1.1.2 Cage2 / Reference 2

During the second 12 month farm cycle cage 2 was left fallow for 4.5 months instead of 3 (see chapter 1.4). No significant interaction between treatment and time was detected, but both factors significantly affected CFB community dynamics (Table 6-2).

Table 6-2 NPMANOVA results for Cage 2/reference 2 CFB community dynamics over stocking cycle 2

Source	df	F	p
Treatment	1	22.82	0.001
Time	2	7.94	0.001
Error	12		

Cage site CFB communities were different from those at reference sites and the trend in change over time is similar over both treatments (Figure 6- 1C). The nMDS results indicate that both cage and reference communities have shifted from those observed at the beginning of farm cycle 2, but remained relatively stable throughout the stocking period.

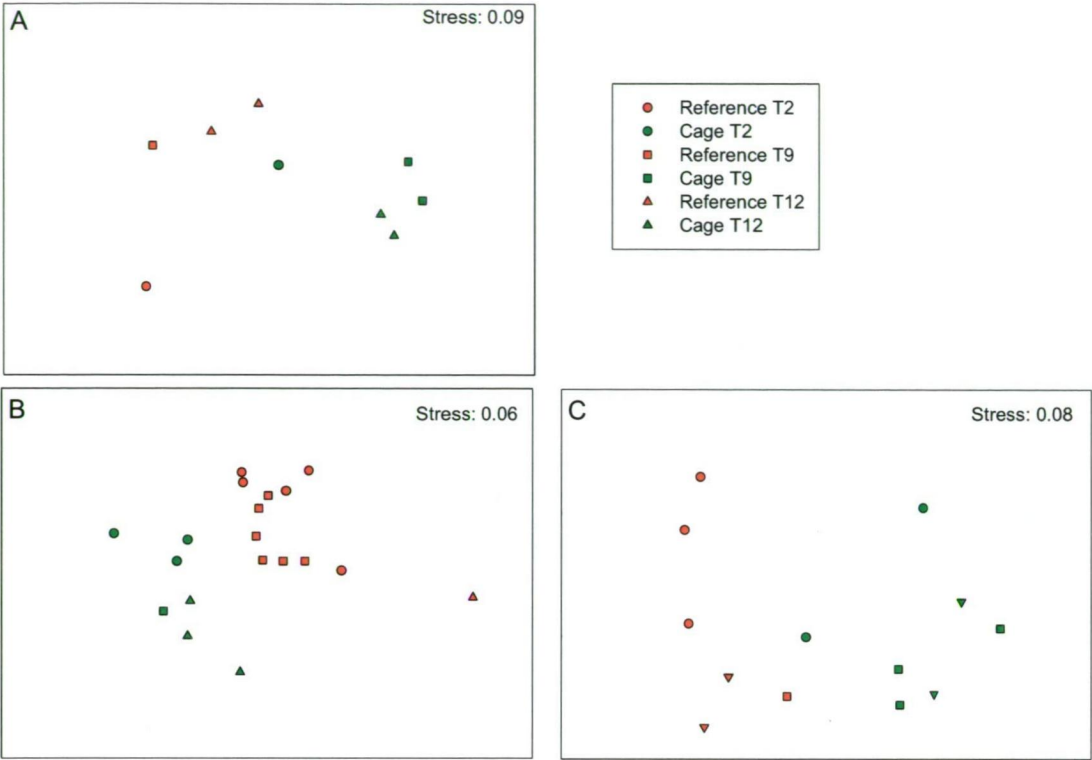


Figure 6-2 MDS plots showing CFB community shifts at farm 1 during the 24 month study. A) Cage and reference sites during stocking cycle 1 (insert dates), B) Cage 1/Reference 1, stocking cycle 2 (insert dates), and C) Cage 2/Reference 2, stocking cycle 2 (insert dates). For A, T2 = 2 months, T9 = 9 months T12 = 12months, for B T2 = 12 months, T9 = 21 months T12 = 24months, for C T2 =13.5 months, T9 = 21.5 months T12 = 25.5 months.

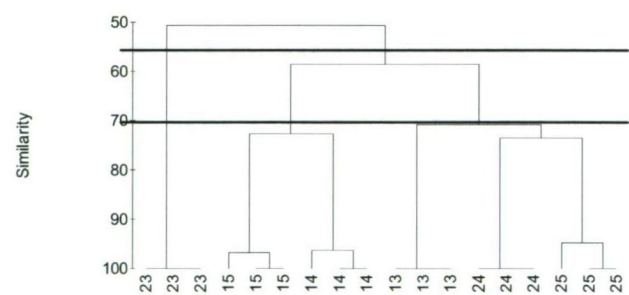


Figure 6-3 Hierarchical cluster diagram of treatment time combinations from cage 1, reference 1 over stocking cycle 2. Samples with a first digit of 1 denote cage sites and those with a first digit of 2 are reference sites. The second digit refers to the time (3 = start of stocking period, 4 = end of stocking/start of fallowing and 5 = end of fallowing)

6.3.2 CFB DNA concentration at farm 1

During the first stocking cycle both treatment ($F_{1,32} = 100.49$, $p = 0.000$) and time ($F_{2,32} = 7.98$, $p = 0.002$) had a significant effect on the amount of CFB DNA in the sediment. More CFB DNA was present in cage site sediment throughout the 12 month period and the amount of CFB DNA increased over the stocking period and declined during the fallowing period. CFB DNA concentrations increased most markedly at the cage sites during the nine month stocked period (Table 6- 3).

During the second stocking cycle at cage 1 and reference 1 both treatment ($F_{1,18} = 125.14$, $p = 0.000$) and time ($F_{2,18} = 16.75$, $p = 0.000$) had a significant effect on the amount of CFB DNA; at cage 2 and reference 2 only treatment ($F_{1,16} = 47.49$, $p = 0.000$) influenced CFB DNA. The CFB population at cage1/reference 1 was larger at the cage site, increased at both cage and reference sites during the nine month stocking period and declined during the 3 month fallow period (Table 6- 3). At cage 2/reference 2 CFB numbers also followed this general pattern (Table 6- 3).

Table 6-3 CFB DNA ($\mu\text{g}/\mu\text{l}$) at farm 1 during the 24 month trial.

Time	Farm Cycle 1		Farm Cycle 2			
	Cage	Reference	Cage 1	Reference 1	Cage 2	Reference 2
T2	0.07 ± 0.013	0.029 ± 0.009	0.068 ± 0.007	0.011 ± 0.0032	0.082 ± 0.011	0.013 ± 0.004
T9	0.186 ± 0.01	0.023 ± 0.004	0.125 ± 0.015	0.019 ± 0.0012	0.129 ± 0.001	0.031 ± 0.015
T12	0.075 ± 0.005	0.010 ± 0.001	0.034 ± 0.005	0.0092 ± 0.003	0.081 ± 0.026	0.007 ± 0.001

6.3.3 CFB diversity at farm 2

Nine samples were omitted from the analysis of CFB community profiles during the first 12 month cycle because no bands were observed on DGGE gels. There was no significant interaction between treatment and time, but the CFB community was significantly different between cage and reference sites ($F_{7,17}=34.7$, $p=0.0001$). Time also had a significant effect on CFB community ($F_{2,17} = 8.9$, $p = 0.002$). ANOSIM showed all times were different from one another (Table 6- 4), but that CFB communities did not change as much during the fallowing, as they did during the first 9 month stocking period (Figure 6- 3).

Table 6-4 ANOSIM results for the effects of time on CFB community profile at farm 2, cycle 1. T2=2 months, T9=nine months, T12=twelve months

Groups	R statistic	Significance level %
T2, T9	0.791	1.1
T2, T12	0.638	0.3
T9, T12	0.331	3.3

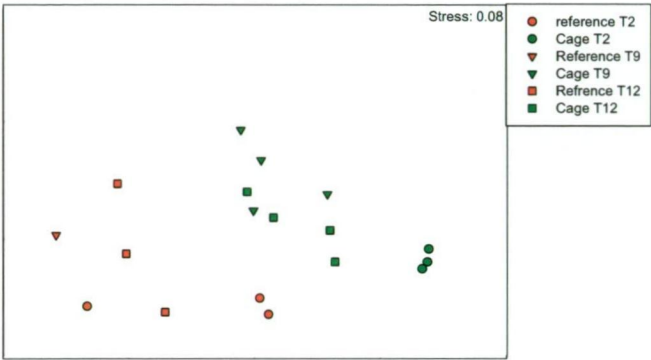


Figure 6-4 Two dimensional nMDS plot of Cage and Reference site CFB community data from Farm 2, stocking cycle 1. Groupings indicated on plot are supported by hierarchical clustering.

During the second farm cycle at Farm 2 nine samples were also omitted. NPMANOVA showed a significant interaction between treatment and time ($F_{2,20} = 3.9, p=0.003$) on CFB community dynamics. Figure 6- 4 shows an nMDS plot of treatment/time combinations for CFB banding patterns. The stress level of this plot is relatively high, but its interpretation is aided by ANOSIM results for comparisons between cage and reference sites at each time and between the cage sites and the reference sites (Table 6- 5). At the beginning of the 12 month farm cycle CFB communities at cage and reference sites were not significantly different. At the end of the nine month stocking cycle the CFB communities at both reference and cage sites had shifted (but in different directions) and had become different. After the fallowing period the communities had shifted again to become more similar. Although the significance value of $p = 0.05$ suggests that the groups were not different, the R value of 0.782 indicates otherwise. Given the low number of permutations and the MDS plot it appears most likely that although the CFB communities at the end of the cycle had shifted to become more similar, they still remained markedly different.

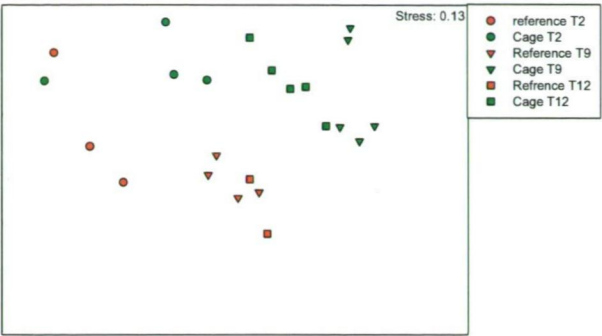


Figure 6-5 Two dimensional MDS plot of Cage and Reference site CFB community data from Farm 2, stocking cycle 2.

Table 6-5 ANOSIM results comparing the similarities between pairs of Cage and Reference sites at Farm 2, stocking cycle 2.

Groups	R statistic	Significance level (%)	Permutations
Cage T0, Ref T0	0.333	11.4	35
Cage T9, Ref T9	0.885	0.2	462
Cage T12, Ref T12	0.782	4.8	21
Ref T0, Ref T9	0.833	0.5	210
Ref T9, Ref T12	0.969	3.6	280
Ref T0, Ref T12	1	6.7	15
Cage T0, Cage T9	0.956	0.5	210
Cage T9, Cage T12	0.045	30.1	462
Cage T0, Cage T12	0.563	1.6	126

6.3.4 CFB DNA concentrations at farm 2

During the first 12 month cycle at farm 2 CFB DNA concentrations were affected by treatment ($F_{1,27} = 13.74, p = 0.001$), but not by time. The amount of CFB DNA was significantly higher ($p < 0.05$) at cage sites. The amount of CFB DNA remained stable at the reference sites throughout the first 12 month cycle (Table 6- 6). During the second 12 month farm cycle at farm 2 both treatment ($F_{1,27} = 5.551, p = 0.028$) and

time ($F_{2,27} = 4.299$, $p = 0.027$) had significant effects on the amount of CFB DNA in sediments. The amount of CFB DNA was higher at the cage sites and increased over the nine month stocking period and declined over the 3 month fallowing period (Table 6- 6).

Table 6-6 CFB DNA (ug/ul) at farm 2 during the 24 month trial. (Mean \pm se, n = 3)

Time	Farm 2 Cycle 1		Farm 2 Cycle 2	
	Cage	Reference	Cage	Reference
T2	0.117 \pm 0.029	0.035 \pm 0.005	0.113 \pm 0.042	0.032 \pm 0.012
T9	0.447 \pm 0.154	0.029 \pm 0.009	0.379 \pm 0.078	0.153 \pm 0.091
T12	0.255 \pm 0.051	0.032 \pm 0.012	0.165 \pm 0.079	0.038 \pm 0.027

6.3.5 Sequences from DGGE gels

All bands excised and sequenced from DGGE gels were found to fall within the CFB group (Figure 6- 5). Most of the sequences found in sediments associated with farm 1 and farm 2 were more closely related to gene sequences from uncultured organisms than to sequences from cultivated species. All sequences did, though, group with other environmental sequences found in a variety of ecosystems, including marine sediments. Also of interest is the fact that several of the band sequences from both farms grouped closely with clones found in the same sediments (labelled Nubeena and Dover in Figure 6- 5), suggesting that they comprised a significant part of the community and that the primer set used (558f and 1392rc) was effective in amplifying the targeted bacterial species.

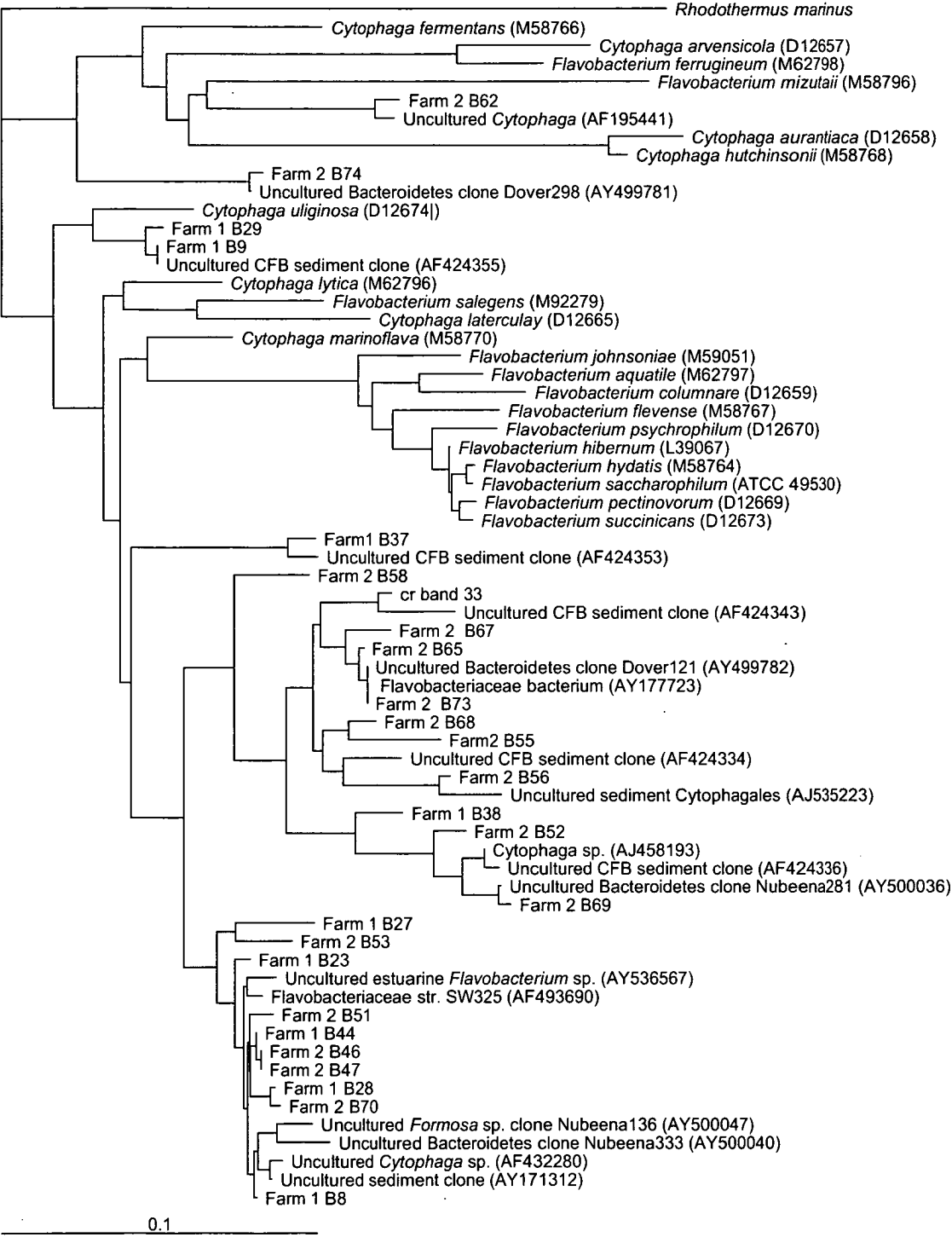


Figure 6-6 Similarity tree showing DGGE band sequences and various members of the CFB group

6.4 Discussion

This study investigated shifts in the CFB community at two salmon farms during commercial farming operations. Farming conditions included two cycles of nine months intensive farming followed by three months fallowing. The study sought to assess farm induced shifts in the CFB community and to assess the efficacy of fallowing in allowing sediments to recover from organic loading. The PCR primers utilised, successfully amplified partial 16S rDNA fragments from CFB bacteria. Although the actual CFB population (both in terms of diversity and density) may have been underestimated ((Kirchman, 2002)) the diversity exhibited by these sequences suggests that the primers did cover much of the CFB population from these sediments (see below). All fragments excised from DGGE gels and sequenced, grouped with CFB sequences.

At farm 1, CFB communities at cage and reference sites were significantly different from one another at each sampling time. This result highlights the rapid response of the CFB community to organic input. The first samples were obtained two months after the commencement of farming. Rossello-Mora *et al.* (1999) reported a rapid response in carbon mineralisation rates and increase in microbial numbers after the addition of organic matter to sediment microbial communities. They also attributed much of this response to CFB group bacteria. The sites at farm 1 also showed much within-treatment variation in CFB community structure over time, to the extent that the reference site communities also changed significantly between sampling times (Figure 6- 1). The effects of temperature on microbial communities and microbial production are well known (Rossello Mora, Thamdrup, Schaefer, Weller, Amann, 1999) and the seasonal variation exhibited in these sediments is not unusual, but does make the interpretation of the farm treatment effect more difficult.

At farm 2 the CFB communities at cage and reference sites were significantly different for all but one sampling time (the start of the second stocking period). Again the first samples were taken two months after farming had commenced. The reference site CFB communities shifted significantly between sampling times. It appears that at farm 2 the CFB communities also exhibit a large degree of natural seasonal variation. Despite the noise created by the large natural variation it is clear

that the CFB community does shift in response to organic loading at this farm (Figures 3 and 4).

CFB numbers at both farms (as measured by CFB rRNA gene amounts) increase with stocking and decrease with fallowing. Although it is possible that the increase in CFB DNA during farming was due to a population shift to CFB bacteriotypes that possess a larger number of *rrn* copies, other studies (Cytryn, Gelfand, Barak, van Rijn, Minz, 2003; Kirchman, 2002; Rossello Mora, Thamdrup, Schaefer, Weller, Amann, 1999) have shown CFB numbers to increase with organic loading and it seems likely that this is the case here. These studies have also suggested that the CFB may play a key role in the initial degradation of complex organic substrates, supplying hydrolysis and fermentation products for further mineralisation. The importance of the CFB group bacteria, performing this initial degradation step, in stimulating benthic microbial production in sediments receiving large amounts of anthropogenic organic input is also suggested here. Inferred CFB numbers increased rapidly at both farms during the stocking cycle, even though sediments became largely oxygen depleted (personal communication, Chris Burke). As the available carbon was utilised over the fallowing period, CFB numbers declined.

CFB communities shift with organic loading at both farms and CFB numbers also follow a similar pattern at both farms; increasing over the farming period and declining during fallowing. Other benthic parameters have exhibited different responses to organic loading, depending on sediment type (see chapters 4.4 and 7). The sediment at farm 1 possessed low organic carbon levels (0.2%), while those at farm 2 were high in organic carbon (4%) (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). This suggests that the CFB were able to adapt to conditions under organic loading well, further supporting the notion that these bacteria are important in organic matter remineralisation, even in anaerobic sediments (Rossello Mora, Thamdrup, Schaefer, Weller, Amann, 1999). When undertaking environmental monitoring it is advantageous to target markers that respond in a predictable manner regardless of external factors such as differences in sediment conditions. More work would be required to use the CFB community in such a manner, but the fact that it responds similarly regardless of sediment type

suggests that limits/regulations formulated with this group in mind may be widely applicable.

All sequences returned from excised DGGE bands grouped with CFB sequences from a wide range of environments (Figure 6- 5). These include Antarctic sediments, anaerobic sulphate-reducing sediments, marine plankton and sediments impacted by hydrocarbon spills. Many of the band sequences also grouped closely with sequences found in clone libraries from these sediments (AY499781-2, AY500036, AY500040 and AY500047) (see chapter 2), suggesting that the primers had good coverage of CFB types from these sediments. The CFB communities at both farm 1 and farm 2 are diverse and appear to overlap considerably. This overlap is discernable by the large spread of sequences from both farm one and farm 2 throughout the entire tree shown in Figure 6- 5.

The molecular techniques employed to assess the CFB community in these sediments have proven to be very useful in long term environmental monitoring. Although DGGE lacks the resolution of large scale clone libraries, the latter are impractical for medium/long term environmental monitoring. DGGE on the other hand, allows the throughput of enough replicate samples to make meaningful assessments of environmental impacts. The same may be said for the real time PCR approach used here. While it may not be possible to definitively enumerate the bacteria under investigation, as may be the case with fluorescent in-situ hybridisation, it is possible to assess relative abundance (impacted versus unimpacted reference) and hence gauge impact. It should be noted that some limitations apply to the statistical interpretations of multivariate DGGE data. If replication is low, the limited number of permutations possible in permutation tests may limit the power of the analysis, making the detection of a significant result at $\alpha = 0.05$ impossible (Kropf, Heuer, Gruning, Smalla, 2004). This limitation can be avoided by ensuring adequate replication. This is especially important in studies where the expected impact will be small. The effects of organic loading from the fish-farms in this study were such that they were detectable despite the low replication sometimes used.

6.5 Conclusions

The results presented in chapter 6 indicate that fish-farm derived organic input is one of a number of factors that influence CFB community composition and that the availability of organic matter is the major determinant of CFB density. CFB communities showed both natural variation over time and as a result of organic loading at both cage and reference sites at both farms. The dynamics of CFB density however, responded quickly to organic matter input and its cessation, although a degree of natural variation did exist.

The CFB communities at both farms appeared to be very diverse and resilient to organic loading. In fact because of the positive response of the CFB to farm waste it is highly likely that these bacteria play a fundamental role in the initial degradation of organic and are important determinants of the system's assimilative capacity.

Chapter Seven

General Discussion

Chapter 7 General Discussion

Estuarine and near-shore coastal areas are important receiving grounds for anthropogenic input and an understanding of microbial community dynamics following perturbation is important to understanding the effects of anthropogenic change on these zones. Sediment bacterial communities facilitate biogeochemical transformations, decomposition of organic matter and the attenuation of contaminants. The nature of bacterial community response to perturbation is however, poorly understood. This study investigated the microbial ecology of aquaculture sediments in order to answer questions regarding bacterial community stability and dynamics and to ascertain the efficacy of current farm management practices in ensuring healthy sediment microbial communities.

7.1 Response of the microbial community to organic disturbance

It was hypothesized that the response of the microbial community would be similar to that shown by other communities when presented with organic disturbance.

Traditionally, sediment health and diversity are monitored by studying the sediment infauna, and the stages that infaunal communities progress through with increasing organic disturbance are described by Pearson and Rosenberg (1978). The infaunal communities in the sediments studied here did demonstrate the expected general response (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004), but the microbial communities did not.

As has been reported elsewhere (for example, (Albertelli, Covazzi, Danovaro, Fabiano, Frascetti, Pusceddu, 1999; Findlay, Watling, Mayer, 1995; Paerl, 1998; Vezzulli, Chelossi, Riccardi, Fabiano, 2002)) bacterial numbers and biomass increase with organic loading, as they did in this study. The elevated bacterial numbers persisted throughout the entire nine-month stocking period, before declining towards reference site levels with fallowing. As organic loading progresses it is usual to see an initial increase in biomass (in both macro and micro-organisms) until conditions become such that they may inhibit the growth of many species. The reasons for this increase in biomass are several. Firstly, there is an increase in a formerly limiting

resource: nutrients. Secondly, succession events occur as opportunistic species that can tolerate the changing conditions rapidly utilize the new resource, out-compete other organisms and grow rapidly. It is expected that biomass will stabilize when nutrients again become limiting or when conditions become too toxic for the growth of the opportunistic species. Bacterial numbers increased throughout the stocking cycle and only declined after cessation of the organic loading, suggesting that the process outlined by Pearson and Rosenberg (1978) may have been proceeding in terms of bacterial biomass, but that conditions did not progress far enough to retard bacterial growth (infaunal communities were not observed to reach Pearson and Rosenberg's (1978) grossly polluted stage (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). Although it is arguable that environments will never become extreme enough to prevent bacterial growth, Macleod *et al.* (2004) concluded that the assimilative capacity of the sediment community (micro and macro) in this study was not exceeded. Even if it is true that bacterial growth will always occur regardless of the environment (for example Kashefi and Lovley (2003) recently isolated bacteria capable of growth at 121°C) bacterial growth rates will diminish as conditions become extreme. The sediments in this study may have deteriorated to a greater extent, and bacterial growth declined, if loading had persisted for longer or was heavier. In terms of controlling microbial biomass and giving sediments the opportunity to process the organic input, it appears as though farm fallowing practices were efficacious, because bacterial numbers and biomass return to ranges close to those observed at the reference sites during fallowing. It also appears that, in these sediments at least, nutrients were the most limiting factor for microbial growth. Although it would appear that a straightforward relationship between nutrients and growth exists, this is not the case. It is well known that sediment infaunal activity can stimulate microbial growth and it remains unclear whether the increased microbial growth in this study can be directly attributed to the increased nutrients, to the activity of opportunistic infaunal species or, as is most likely, to a combination of the two. What is certain, however, is that microbial growth was stimulated, directly or indirectly, by farm loading and that this aided in the sediment's ability to process added organic matter.

The assessment of the effects of organic perturbation on microbial diversity and how these relate to the processes outlined by Person and Rosenberg (1978) is not as simple

as the above interpretation of microbial biomass dynamics might indicate. Bacterial diversity was assessed by both clone library and genetic fingerprint techniques. Clone libraries demonstrated the immense diversity of these sediments, both at farmed and unfarmed sites. All libraries were shown to be different and although it is tempting to attribute the apparent changes in bacteria at the farm sites to organic loading this may not be the case. DGGE results show that the bacterial communities at both farms and reference sites are dynamic, that farm site communities shift with changes in organic load and that reference site communities shift with some as yet undetermined, seasonal factors. There was no temporal aspect to the reference site clone libraries; therefore it is not possible to attribute changes in farm site libraries to organic loading alone, even though it is likely that organic loading was a major contributing factor to these changes.

DGGE fingerprint analysis demonstrated that bacterial diversity responded to organic loading, or more correctly that the bacterial communities' dominant players shifted in response to increased organic nutrients. The shifts in total bacterial community (DGGE with universal primers) demonstrated the high functional redundancy of bacterial communities in that although shifts occurred, they were not of the simple shift/counter shift variety often seen in sediment infaunal communities (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004; Pearson, Rosenberg, 1978). In the sediments of this study the infaunal communities were shown to shift with organic load and counter-shift (communities returned some way toward those observed pre-disturbance) during fallowing (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). In the bacterial communities however such was not the case. Bacterial communities were observed to shift at each sampling time, but community shifts did not show a cyclical nature with either change in farm stages (cage sites) or seasons (reference sites). The reason for this may be the high functional redundancy of bacteria. The bacterial community appears to be determined by random colonization processes (Reice, 1994), rather than by predictable succession events. The number and phylogenetic type of bacteria present that can fill any one niche is potentially very large and as the sediment undergoes rapid change niches are filled at random. At cage sites this rapid change is seen as farming and fallowing process, but would include the "disturbances" seen at reference sites including, seasonal temperature changes, storm and sedimentation events,

bioturbation, predation etc. These processes manifest themselves on DGGE fingerprints as a constantly shifting of the community. If predictable succession events dominated, then sediment communities at reference sites would have remained constant and those at farm sites would have approached an alternative stable community at the end of the farming period, and returned toward the original community on fallowing. Such a pattern was evident in infaunal communities in these sediments (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004), to the extent that communities became more similar at each farm after farming, despite different intrinsic sediment characteristics, but not in microbial communities.

The AOB were used in this study as a keystone species; that is one that performs an important ecological role that will not be fulfilled by any other species. In this case the AOB are thought to be intolerant of the conditions associated with fish-farm loading (Kowalchuk, Bodelier, Heilig, Stephen, Laanbroek, 1998; McL. Macdonald, 1986) (discussed in chapter 5.1) and perform the role of nitrification, important to the coupled nitrification-denitrification process. Although the functional redundancy of bacteria filling the aerobic nitrification niche is thought to be relatively low (in that few species perform the role), the DGGE band sequences have shown a large amount of sequence diversity within this group in these sediments. This and the fact that AOB communities were continually shifting as sediment conditions changed, suggests that this group is resilient and can adapt to perturbation. The fact that the AOB were considered a keystone species in this study may be erroneous for several reasons. In fact, the whole concept of keystone species in complex bacterial communities may be misplaced. The AOB are very difficult to cultivate and it is likely that the diversity of this group is far from appreciated. It may be that although there appears little redundancy in this group in terms of the organisms capable of oxidizing ammonia, the group may possess great redundancy in terms of traits allowing its members to persist under a variety of conditions. The beta-proteobacterial AOB have recently been shown to exist in anoxic sediments (Freitag, Prosser, 2003; Mortimer, Harris, Krom, Freitag, Prosser, Barnes, Anschutz, Hayes, Davies, 2004). Mortimer *et al.* (2004) used RT-PCR to demonstrate that the AOB found deep within the anoxic zone of their sediments were active. Using pore water chemistry techniques they also inferred that the AOB were carrying-out anoxic nitrification processes. Despite parallel oxygen

microelectrode studies demonstrating apparent anoxia in the sediments of this study (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004), the dynamic nature of the AOB communities sampled here suggest it is likely that the nitrification process is occurring. It remains to be elucidated whether nitrification is occurring aerobically or anaerobically. It is likely that microenvironments containing oxygen exist in the sediment despite it appearing uniformly hypoxic. The sediments in this study experienced considerable bioturbation, which may have helped maintain oxic-zones and facilitated the aerobic nitrification process. Finally, the recent discovery (Kuypers, Sliekers, Lavik, Schmid, Jorgensen, Kuenen, Damste, Strous, Jetten, 2003) of organisms capable of the ANAMMOX reaction suggests that oxidation of ammonia could proceed without the AOB. Several clones did indeed, group with sequences associated with phylotypes thought to capable of the ANAMMOX reaction. It would appear then, that ammonia oxidation and denitrification (either directly via ANAMMOX, or via anaerobic nitrification and the subsequent denitrification of the oxidized products) are likely to proceed in organically loaded sediments such as those studied here, despite a strong perceived association of this process with aerobic conditions. More research is needed to answer the question of the validity of the keystone species concept in complex bacterial assemblages, but in these sediments at least it appears as though the AOB community remained healthy despite organic loading and indeed was able to respond to it. In order to answer these questions and assess the effects of organic loading on nitrogen cycling, rather than simply on the diversity of the organisms responsible for it, it is necessary to measure nitrification rates and pore water chemical gradients in response to organic loading and relate these measurements to the bacterial species present that may be responsible. A task that can be undertaken, now the identities and diversity of many AOB organisms inhabiting these sediments are known.

Another interesting finding is the dynamic nature of all reference site bacterial communities studied. It was thought that these communities would be relatively stable and exhibit some of the characteristics of communities approaching climax. The fact that this is not the case supports the idea that complex bacterial communities, such as those in sediments, are undergoing constant change in order to respond to and mould their environment. Most studies in microbial ecology utilize single sampling times and attempt to look at different environmental parameters along some type of

gradient. It is unclear, though, whether community changes in such studies are attributable to the different conditions or simply to the heterogeneous and dynamic nature of these complex communities.

In this study the CFB group bacteria were classified as an opportunistic group for the purposes of assessing bacterial response to organic loading. It was expected that these opportunists would increase in number as loading progressed and decline during fallowing, as was observed for the opportunistic Capitellid worms in these sediments (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004). The CFB did exhibit this expected response and also showed continued shifts in community structure during the trial. Heterotrophic bacteria such as those from this group are no doubt important in the rapid response of bacterial communities to organic input (Cytryn, Gelfand, Barak, van Rijn, Minz, 2003; Rossello Mora, Thamdrup, Schaefer, Weller, Amann, 1999). The concept of an opportunistic species though was not necessarily upheld in these sediment bacterial communities.

Opportunistic species not only colonize rapidly, but also dominate communities by out-competing resident species and eventually contribute to a loss of diversity. This pattern is clearly seen in the Capitellid spp. dominance of organically polluted sediments (Pearson, Rosenberg, 1978). Although the CFB did increase as a proportion of sequence types in the F1C9 clone library, they did not come to dominate any library to any great extent. The number of different CFB sequence types detected actually increased, even though other sequence types detected decreased as CFB sequences increased. The CFB are instrumental in maintaining benthic production (Cytryn, Gelfand, Barak, van Rijn, Minz, 2003; Rossello-Mora, Thamdrup, Schaefer, Weller, Amann, 1999), but do not come to dominate the community as opportunistic infauna such as *Capitella* spp. do. Instead the CFB are important initial degraders and supply hydrolysis and fermentation products to other bacterial groups. Their ability to rapidly respond to organic loading has been reported elsewhere (Cytryn, Gelfand, Barak, van Rijn, Minz, 2003; Rossello-Mora, Thamdrup, Schaefer, Weller, Amann, 1999) and is demonstrated here.

From the above discussion it would appear that the bacterial community is too complex to monitor in relation to perturbation in any straightforward way, and that despite perturbation it is likely to remain diverse. Indeed this idea has been espoused

previously (Curtis, Sloan, 2004). Microbial communities though are a major driver of sediment function and this function has effects far up the food chain; therefore it is necessary to understand microbial community dynamics if we are to manage marine in-shore resources in a sustainable way. The way in which bacterial communities do not appear to follow the equilibrium theory, but are determined randomly, also casts doubt on the validity of the keystone and opportunistic species concepts in microbial ecology. Closer examination though, may reveal that these concepts simply need more development. The concept of a keystone species (or guild) may be better applied in terms of maintaining desirable rates of key processes, rather than simply maintaining the diversity necessary for the process to occur. After all, when considering complex dynamic systems and their response to perturbation we are really assessing the system's ability to maintain some arbitrary value placed on it by the observer (for example, trophic state). Such responses are often easily indicated by changes in diversity higher up the food chain (for example infauna), but it is really alterations in microbially-driven process rates that we are often concerned with. It is for this reason that assessing microbial diversity and function is important. As more information comes to light on which phylotypes inhabit complex systems and how communities shift, it is possible to gain an understanding of how anthropogenic inputs can influence microbial processes. For example, now that it is known that AOB communities are very dynamic in responding to environmental change, that they appeared to survive anoxic conditions and that ANAMMOX organisms were also present in the study, it is possible to investigate how; 1) rates of nitrification are influenced by organic input, 2) which organisms are responsible for carrying out the process under varying conditions and 3) how these factors affect denitrification. It is the maintenance of the nitrification-denitrification process that is seen as important in removing nitrogen and preventing changes to a system's trophic state, not the diversity of the organisms that carry out the processes involved *per se*. It is necessary to have knowledge of both the organisms responsible and process rates, if the true assimilative capacity is to be understood and utilized. To this end studies of microbial diversity may be simply the first step in understanding complex systems.

The composition of sediment bacterial communities appears to be influenced by random determinants and do not appear to follow the expected pattern as expounded by the equilibrium theory. Further research is needed to elucidate the exact nature of

microbial community dynamics as they pertain to traditional ecological theories. This research could include the utilisation of simpler communities in microcosm experiments. Such experiments could investigate the questions of unlimited functional redundancy, the nature of recolonization and recruitment in shifting communities, the influence of bioturbation and the rates at which key biogeochemical processes are carried out under varying conditions and by various phylotypes. The utilisation of microcosms would also allow the addition of far more substrate than would be possible in a natural system like the one studied here. It may be that bacterial systems must undergo further shifts to demonstrate similar, predictable responses to those observed in sediment infaunal communities. In terms of using microbial community dynamics to monitor the impact of anthropogenic pollution, it would appear that it is necessary to target specific phylotypes and processes that will have an undesired impact on higher trophic levels, rather than monitor bacterial diversity *per se*. Again an initial understanding of sediment communities and their composition is needed to achieve this end.

7.2 Efficacy of farm fallowing practices in limiting the impact of organic loading on bacterial communities

Fallowing appeared efficacious in maintaining sediment bacterial diversity in that the community shifted during fallowing and other parameters (chemical and infaunal communities (Macleod, Bissett, Burke, Forbes, Holdsworth, Nichols, Revill, Volkman, 2004) suggested that the sediments returned towards pre-farming conditions. The real case may be that sediment bacterial communities remained diverse despite fallowing and that fallowing appeared to make a difference because of shifts in communities, which were also evident at reference sites. Sediment bacterial communities are constantly subjected to disturbance, not just of an anthropogenic nature. They experience storm events, flood events, natural organic matter deposition and bioturbation regularly. The disturbances applied by farms in this study were short and may have been seen as simply another event in a very dynamic system. Fallowing time did however, appear important in terms of allowing bacterial biomass to recover. Bacterial numbers responded rapidly to both farm loading and its cessation.

Sediment bacterial community assessment may not be appropriate for routine monitoring of anthropogenic impact, even though an understanding of microbial dynamics is important in managing such impacts. Emmet Duffy (2003) uses the term “trophic skew” to state that extinction effects are felt most higher up the trophic scale, and noticed less at lower trophic levels. It may be the case that microbial communities are at a trophic level below which we can currently detect meaningful changes in whole communities, in terms of the classic impact response, while the sediment infauna are not: making them a more easily utilized tool for assessment.

7.3 Conclusion

All sediments surveyed in this study were shown to contain a very diverse population of bacteria and although a reduction in sediment bacterial diversity has been observed in aquaculture sediments previously (Torsvik, Daae, Sandaa, Ovreas, 1998), a marked decline in diversity with farming was not observed here. The extremely high functional redundancy of bacterial communities ensures that all niches are filled despite perturbation. This is especially true of sediments such as those studied here that underwent only short term and rapid changes and never approached extreme conditions. Organic nutrients, not metabolic pathways available for its utilization, appeared to be the main limiting factor to bacterial growth.

Sediment bacterial communities and biomass shifted with organic loading and its cessation, but also appeared to be very dynamic naturally. Fallowing appeared efficacious in maintaining a diverse bacterial community and in allowing microbial biomass to reduce to natural levels as organic substrates were utilized. The CFB were strongly linked to the initial degradation of organic matter from farms and despite reduced conditions, the AOB appeared to persist, maintaining at least the potential for nitrification.

The exact nature of bacterial community response to change and how this relates to our macro-ecology derived concepts is unclear, but it would seem that niches are randomly filled from the massive pool of potential candidates, rather than following predictable succession events. This finding and the inherent dynamism of sediment

bacterial communities has implications for the interpretation of community fingerprint data. It is likely that monitoring bacterial communities is not an efficacious means to monitor anthropogenically induced system changes. It is though, important to understand the nature of bacterial system responses in terms of the effect they will exert on processes deemed important to maintaining desired system attributes (for example, denitrification). Although primary studies need to explore bacterial diversity more generally, future studies should target essential ecosystem services and the bacterial communities that mediate them, rather than monitor bacterial diversity *per se*. The coupling of knowledge of bacterial diversity and function and the effects of perturbation on process rates is necessary for the maintenance of system characteristics deemed to be important.

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